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Into the 1980s

CHANGES of Editor at *Nature* are relatively rare: my departure at the end of 1979 will only be the fifth such change in 110 years (admittedly the figures are somewhat distorted by the extraordinary 50 years which the first editor, Sir Norman Lockyer, served). A new editor has yet to be appointed; for the interim Peter Newmark, at present Deputy Editor, will take over. Dr Newmark has had overall responsibility for the selection of biological manuscripts for almost six years and has done this job with distinction. *Nature* will be in excellent hands.

The production of the journal, week by week, year by year, is a collaborative enterprise involving a wide variety of people. It requires the continuing support and encouragement of the board of directors; it requires the endless attention to detail of typesetters, printers and blockmakers; it requires the enthusiasm of advertising, promotion and circulation departments; it requires a lot of hard work from our secretarial and artwork teams. For this I have good cause to be very grateful. And yet my most profound thanks must go to the two groups of people, one small, one large, which between them ensure the continuing vitality of the journal: the editorial staff and a small army of outside helpers.

Tributes to staff can so easily be perfunctory; this one is very heartfelt. My fifteen colleagues who between them decide what goes into the journal, when and how, are a quite outstanding and thoroughly professional team, whose clear appreciation of what *Nature* ought to be and how *Nature* ought to change has been a constant support to me. Maintaining *Nature*'s standards calls for a particularly high degree of dedication to work which can at times be exceptionally onerous. The editorial team possesses this dedication in great measure.

And yet all the in-house ability would count for nothing if we could not depend on scientists from all disciplines and all parts of the world for willing help. One of the interesting things about *Nature*, is that this week's reader may be next week's author, adviser, referee or correspondent. The journal belongs more to a community than to a company, an editor or an editorial team. And without the continued encouragement, help and criticism we get from the scientific community, the journal could not survive. We thank you, the scientist, even though occasionally we choose to ignore your advice or refuse to publish your contribution!

ALL manner of scientific and science-political themes rise and fall during an editor's tenure, and perhaps it is foolish

to single one out for a last comment. After all reams could be written about the growth of public participation in science-policymaking, the problems of a career structure for scientists, the growing awareness of the vulnerability of the scientist to human rights violations, the place of scientists in development, risks to science funding in a weak economy and so on. Yet if there is one issue which consistently fails to capture headlines yet which ought to interest the scientific community, it is that of arms control and disarmament.

The 1970s has not been a hopeful decade. SALT 2, still not ratified, took an extraordinary time to negotiate and, as far as one can see, does little but confirm existing levels. And in order to sell SALT to the American people, other vigorous armaments programmes are promised. A comprehensive test ban treaty, on which hopes were raised recently, is now unlikely to appear in the near future; if ever it does emerge it is going to be so circumscribed as to be meaningless. The Non-Proliferation Treaty, due for review in 1980, has acquired few new signatories whilst a novel brand of proliferation has emerged, as practised by Israel, South Africa and Pakistan — proliferation by assumption. Multilateral Balanced Force Reduction talks in Vienna are hopelessly bogged down.

There is little progress towards a chemical weapons treaty, whilst there are growing fears of chemical rearmament. Meanwhile arms sales to the developing world continue with the utmost vigour, and nuclear nations improve their arsenals not so much quantitatively but qualitatively. And new weapons, such as laser and particle-beam devices against ballistic missiles get expanded research budgets, regardless of the enormous difficulties there will be in converting ideas into hardware.

Scientists should have a lot to say, and publicly, about the technological arms race. After all in most countries ministries of defence are the nation's largest single employers of scientists and technologists. And yet in recent years the enthusiasm that there once was for asking pointed questions on the assumptions behind military expenditure has largely dissipated — no doubt because of the thankless nature of the task and the seeming impossibility of deflecting inexorable trends.

Will any measures of real self-restraint emerge in SALT 3? Are nuclear weapons bound to spread indefinitely? Is there no way of diverting the resources devoted to military purposes into more constructive channels? These are questions for the 1980s . . . and beyond.

David Davies.



Energy shifts meet environmentalist protests

Moves to convert power stations from oil to coal are generating controversy about the effects on air quality. **David Dickson** reports on one such dispute in New York City.

In a classic trade-off between energy and environmental concerns, New York city last week approved a temporary waiver of its air pollution controls to allow a local power company, Consolidated Edison to burn fuel with a greater sulphur content than is currently permitted.

The company hopes to be able to demonstrate that it is able to use high rather than low sulphur content fuels at its power plants in the city without exceeding federal air pollution standards, and that this will make it possible for the plants to convert from oil to coal, a move supported by President Carter as part of his strategy to reduce dependence on foreign oil.

Local environmental and community groups, however, are protesting against the proposal, which has yet to be given official approval by the federal Environmental Protection Agency. They argue that current controls permitting only the burning of oil with a low sulphur content have been one of the most effective anti-pollution measures in the city's history — and that moving back to higher sulphur content fuels could eliminate some of the gains of the past decade.

Con. Ed. has for several years been trying to get permission to increase the sulphur content of the fuel burnt in its power plants. It claims that not only would conversion to coal be in line with administration policy, but that with escalating oil prices, it would also be financially beneficial to customer companies.

In the previous year, the company's application has been turned down, largely on the grounds that the company has not been able to demonstrate that the current policy results in any particular hardship. Since then, however, the political climate for such decisions has changed dramatically.

In his energy message to Congress in July, for example, President Carter stressed his support for power companies converting from oil to coal. He is expected to submit to Congress soon measures costing even more than the \$5 billion that he originally proposed to stimulate the use of coal.

A number of utilities have already benefited from the new climate. In Massachusetts, for example, the New England Power Company, after a four-year battle against stiff local opposition, recently obtained permission to convert three out of four units of a 1,650 megawatt plant from oil to coal, claiming that this would reduce oil consumption in the region by 2.5%.

Last year the company was given permission for an experimental period to burn oil with double the previously permitted 1.1% sulphur content. Company officials admit that pollution levels increased as a result — but point out that they were still within federally-permitted levels.

The current application from Con. Ed. would allow the company to increase the sulphur content of its fuel from 0.3% to 1.5%. It claims that converting three power plants from oil to coal would reduce the nation's dependence on oil by 15 million barrels a year — and would also reduce its customer's bills by 150 million.

"The reason for requesting temporary permission to burn high sulphur content oil is to demonstrate the validity of our contention that such a move will not violate federal primary air quality standards", a spokesman for the company said last week.

Permission for a temporary waiver from current controls has already been given by the New York State Department of Environmental Conservation, and by the New York State Department of Environmental Protection. The application will soon be published for public comment by the EPA, and a final decision is expected by next March or April.

Con. Ed.'s application is being strongly resisted by environmental and community groups in New York. "The City's current requirements that only clean fuels — that is low sulphur content oil and natural gas — can be used has been one of the most successful pollution control strategies that the city has had, and the sulphur content in the air has dropped by 40% since the controls were introduced in 1972", says Marcy Benstock, Director of the New York Clean Air Campaign.

Ms Benstock claims that a comparable reduction in dependence on foreign oil could be achieved through an intensive energy conservation programme. She quotes evidence from a report by researchers at Harvard University that the US could consume 30% to 40% less energy than at present and still enjoy the same standard of living.

Furthermore, she claims, the apparent reduction in costs could be eaten away by additional medical bills and other damage caused by increasing pollutant levels. Even pollution control devices, she argues, will not be able to remove all the additional pollutants caused by converting to coal, such as some of the toxic trace elements.

A new "wrinkle" as one EPA official puts it, recently to have entered the debate, is concern over the extent to which



Smog in New York city: will Carter's energy policy make it worse?

increased sulphur emissions could contribute to a problem receiving growing attention in the US, namely acid rain.

According to a spokesman for Con. Ed., the company does not consider this to be too much of a problem since "by and large the sulphur emissions go out to sea". Others, however, argue that not enough is yet known about transportation patterns to judge the potential degree of the problem.

"At present the burden tends to be on society to show that such things are harmful. But the alternative is equally rational, namely that increased emissions should not be allowed until it can be clearly shown that they are not harmful", says Dr Ellis B Cowling, a plant pathologist from North Carolina State University who has studied the acid rain problem, and gave evidence at an earlier hearing on Con. Ed.'s request.

The transport of pollutants is one of the subjects to be examined in a regional study of metropolitan New York and the adjacent areas of New Jersey and Connecticut that has just been announced by the National Commission on Air Quality.

The company's application to use higher sulphur content fuels is being opposed not only by local groups such as the New York statewide Senior Action Council, and People Outraged With Energy Rates (POWER), but also national groups such as the Sierra Club and the Natural Resources Defense Council.

So far, however, their protests have made little impact, and with the current crisis in Iran bolstering programmes for domestic energy production, as well as an administration keen to help power companies comply with federal legislation in the least burdensome way — and a relative lack of financial and human resources compared to those seeking the application — their chances of stopping Con. Ed.'s plans seems increasingly slim. □

UN agrees on science and development centre

AFTER three weeks of intense debate, member nations of the United Nations agreed late last week to set up a new policy unit to help carry out the recommendations of the Conference on Science and Technology for Development (UNCSTD) held in Vienna in August.

The new unit will be known as the Centre for Science and Technology for Development. It will be headed by an Assistant Secretary General, and is expected to involve between eight and ten new staff appointments, as well as absorbing some of the resources of the present Office of Science and Technology, which is to be abolished.

The main function of the new centre will be to service the Intergovernmental Committee which, it was agreed at Vienna, should be set up to attempt to coordinate the various programmes within the UN system concerned with applying science and technology to development, and to stimulate further activities in this direction.

A resolution calling for the setting up of the centre was finally agreed in the early hours of last Friday morning by the second committee of the UN general assembly, currently meeting in New York.

In their final form, the institutional arrangements are broadly in line with proposals made by the Group of 77, on behalf of the developing countries, that a strict interpretation of the agreement reached in Vienna implied the creation of a new secretariat.

A number of industrialised countries, and in particular the member countries of the European Economic Community, argued strongly that no new institutional arrangements should be made — and that any new functions could be carried out by existing machinery under the Department of International Economic and Social Affairs.

In the end, however, it proved impossible to devise a compromise formula which all sides could agree upon. And the decision to establish the centre — which will report to, but not be under the direct control of, the Director General for Development and International Economic cooperation, Mr Kenneth Dadzie — was put to the vote.

The result was 97 votes in favour, none against, and 20 abstentions. Countries abstaining include members of the EEC and the countries from the eastern European bloc. Those who voted in favour included not only the developing nation members, but also the United States, Canada, and the Scandinavian countries.

The outcome had been held up pending the resolution of a dispute over how much direct influence UN member states should have over the way in which the United Nations Development Programme disburses the "interim fund" which the Vienna conference agreed should be set up

prior to the establishment of a long-term science and technology financing system.

The Group of 77 submitted a document arguing that there should be direct involvement of the IGC — which is open to all UN member states — in the administration of the fund. The developed countries, however, and in particular the US which is likely to be a major contributor, made it clear that such a decision would prejudice their agreement to contribute.

The developing countries agreed to back down on their demands, and responsibility for its management will lie in the hands of the administrator and the governing council of the UNDP, although operating within broad policy guidelines laid down by the IGC.

(A pledging conference for the interim fund will be held early next year, and although it seems that the \$250 million target agreed in Vienna will not be reached, UN officials are optimistic that voluntary contributions totalling about \$100 million can be raised for the two years 1980–1981.)

State Department officials in Washington later expressed satisfaction with the final outcome of the second committee's discussions, claiming that a new centre headed by an assistant director general (some developing countries had argued for an under secretary general) was "just right" — and that to have accepted the Group of 77's proposals for the administration of the interim fund would have destroyed many of the gains made in Vienna.

US Congress approves 13% growth in military support for basic science

THE US Congress last week agreed to increase military support for basic research by 13% in the fiscal year 1980, the Senate in particular expressing its support for the Department of Defense's efforts "to overcome years of real decline in funding for this type of research".

The increase, which will bring support of basic research by DOD to about \$500 million a year, is less than the 16.7% growth initially requested by President Carter. However, in line with changing social priorities, it is considerably higher than the increases in support for basic science by other federal agencies, for example, the 8% growth for the National Institutes of Health.

Overall, Congress agreed to an 8.5% growth in the Defense Department's spending on "research, development, testing and evaluation", from \$12.3 billion to \$13.4 billion. Much of this increase will be allocated to support of the "technology base", which includes research on high energy systems, such as lasers and particle

How the US should pursue the outcome of UNCSTD is likely to be one topic discussed by a new advisory panel on science and technology for development which is being set up under the chairmanship of Mr Tom Pickering, assistant secretary responsible for the Bureau of Oceans, Environment and Scientific Affairs.

The panel is being established under the aegis of the bureau's advisory committee, and its first meeting is likely to be held in public. Additional topics that have been proposed for discussion include energy and development, and a forward look to the global picture in the year 2000.

Meanwhile administration officials are still trying to salvage plans for an Institute for Scientific and Technological Cooperation (ISTC) a centrepiece to US presentations in Vienna, but for which funds have been refused by the Senate.

The House of Representatives has agreed that funds for the institute should be provided. But so far a joint House/Senate conference, which is bogged down on a number of foreign aid issues, has failed to agree on whether funding for the institute should be provided.

One proposal currently being studied is that the ISTC should not, as initially envisaged by the administration, exist as a separate body within the US aid programme, but remain under the administration of the Agency for International Development, many of whose research programmes the ISTC would be taking over. **David Dickson**

beams, as well as high speed integrated circuits.

In particular, support for R,D,T and E activities within the defense agencies — responsible, for example, for the Defense Advanced Research Projects Agency (DARPA) — will grow by 8%.

University research workers are likely to be a beneficiary of the increased spending. Although last year Congress refused to establish a proposed "defense science and engineering programme" aimed at increasing the effectiveness of relationships between the DOD and university scientists, it has given encouragement to the department's efforts in this direction.

The Senate in particular has given support to an increased emphasis on basic research within the R and D budget. Although the House of Representatives had made several cuts in the President's budget request, many of these were restored by the Senate, and subsequently accepted at a conference between the two houses. □

Italy threatens to disrupt three key European research institutions

Italy has drafted a career diplomat to its Ministry of Science expressly to improve its position in European research organisations. **Robert Walgate** reports from Rome.

ITALY was last week preparing to "dissociate" itself from CERN, the European organisation for nuclear research; to block (for a second year) the budget of the European Space Agency; and to refuse to fund JET, the Joint European Torus for fusion research under construction at Culham, England, for a further four-year term.

The three questions were to be raised at meetings of the council of ESA on 18 December, CERN on 19 December, and the Council of Research Ministers of the EEC on 20 December. So Vito Scalia, Italy's Minister for Scientific Research, told *Nature* in an exclusive interview last week.

At CERN, said Scalia, Italy would "consider very seriously" adopting merely an observer status if the appointment of a new director-general were not postponed until April, and if there was not "satisfactory clarification of the future programme of CERN".

Italy is undoubtedly the most concerned of all countries at Germany's precise intentions towards CERN, since it emerged that Germany may build a 6-km electron-position accelerator — HERA — at Hamburg. HERA is felt by some to be a competitor for CERN, which hopes to build a similar (but bigger) ring in the 1990s — LEP. Germany denies that LEP and HERA are competitive (see *Nature* 6 December p 546).

But since Germany pays the ceiling of 25% of the CERN budget, and CERN costs and salaries are high, it would arguably be cheaper for Germany to build its own LEP on home territory; hence Italy claims its fears may not be groundless.

Italy's view is also complicated by the fact that of the two principal candidates for the director-generalship of CERN one is the German director of the laboratory where HERA may be built — Professor H. Schopper — and the other is the leading Italian physicist Professor A. Zichichi. Schopper's appointment at CERN "would be rather like the entry of a Trojan horse", said one Italian official. "Someone should be appointed whose Europeanism is not in question".

In contrast to this view, however, Professor Schopper has always insisted that his laboratory at Hamburg be considered an international institution. At PETRA, presently its largest accelerator and a world leader, he refused to admit groups composed of one nationality. This at one stage excluded Italy, which wanted to run a massive prestige single nation experiment at PETRA. At present there is

no Italian group at PETRA, despite the fact that less than half PETRA's experimenters are German. German-Italian relations are thus somewhat tender on this issue.

At the European Space Agency, Italy will re-affirm its year-old decision to block the ESA budget because, says Scalia, "there is insufficient industrial return to compensate for our £10 million losses over two years on Spacelab". Here Italy believes that ESA's professed policy of distributing contracts among members in proportion to their contributions is manifestly not working. "Germany underwrote Spacelab 54%, Italy 18%", said a ministry spokesman. "But Germany got 62% of the contractors, ourselves 10%".

Scalia also criticised France and Germany for failing to contribute to 'L-sat', ESA's venture in Europe-wide television transmission. France and Germany are bi-laterally developing the equivalent 'TV-sat', leaving Britain, Italy, and five other countries alone in the ESA project.

Italy is also interested in re-organising ESA when the present director Roy Gibson completes his term. "The programme lacks momentum and imagination" said an official. According to Scalia, ESA "should find a strong manager".

In Brussels, Italy would like to link the future of the European nuclear fusion experiment, JET, with that of 'Super Sara' — an experiment on the core 'melt-down' of thermal reactors which would take place at the European Commission's joint research centre at Ispra, north Italy. Britain and other delegations have consistently refused to fund the £35 million experiment, ostensibly because they feel that the costs are too high.

"It would be interesting to know" said Scalia "in what way the countries that oppose such an important programme on reactor safety — one which has been described by America as of great significance — will justify their position to the public . . ."

Germany, an Italian official told *Nature*, had already approached Italy to see if the experiment could be undertaken bi-laterally. Japan has also shown interest. "Whoever can develop a demonstrably safe reactor, will take the world market" said the official. But Italy would prefer that the experiment be undertaken under a pan-European banner.

Criticism of Super Sara has centred on the efficiency of Ispra staff. "We reject that" said the official. Encouraged by the government, Italian unions had sent

observers to Ispra, to Brussels, and to Culham where JET is being built, "to assess absenteeism, times of arrival for work and so on". The results put Ispra ahead of Culham, and firmly ahead of Brussels, he said.

"We must have a European programme on nuclear safety" said Scalia. "And at Ispra we have a most important establishment both in the quality of its staff and its equipment." JET and Super Sara, he said, "must either stand or fall together".

Italy appears to be strengthening its stand on European scientific and technological issues. With the change of government four months ago a career diplomat was appointed to the Ministry of Scientific Research as 'chief of cabinet' — the principal advisor to the new minister — and he has been playing an active role in asserting Italy's position in Europe.

The objectives of this exercise may not be entirely a matter of supporting Italy's scientists. The country has taken a strong interest in the industrial aspects of space, having recently announced a £125 million five-year national programme ("fully complementary to ESA's"), and is mindful of the possible applications of the superconducting technology that will be needed in the long term for high energy accelerators. The proof of nuclear safety is related both to sales of reactors and to convincing Italy's public that nuclear power is acceptable.

"Advanced technology" said Scalia "must be a European problem". □

Call for European environment charter

EUROPE'S environment ministers should convene a conference on the environment some time during Italy's forthcoming presidency of the Council of Ministers, Italy announced at a council meeting at Brussels on Monday. The meeting would demonstrate to environmental groups that governments were prepared to tackle environmental problems, said Italy's science minister Vito Scalia last week.

There should also be agreement on a charter announcing the principle of "polluter pays" said the minister, and ultimately, "no pollution".

However, Italy's own record on pollution control is not outstanding. The United Nations Regional Seas Programme, a component of the UN Environmental Programme, recently advised Italy to lower its standards for pollution of bathing beaches — because the existing standards were impossibly high. "If Italy applied them, all their beaches would be closed" said a UNEP spokesman. "Better to take a realistic level, and then actually apply it."

Europe's new space launcher stays on the launch pad

ARIANE, Europe's 200-tonne 3-stage launcher designed to put 1-tonne payloads into geostationary orbit, stayed resolutely on its launch-pad last Saturday during the first attempt to put the vehicle into space. Journalists, watching closed-circuit television screens at the European Space Agency's headquarters in Paris gripped their notebooks excitedly, and radio reporters cleared lines to their news bureaus, as the seconds ticked away to zero. With a few seconds to go, the two "cryogenic arms" which had fuelled the rocket in the previous hours and minutes swung away; there was a cloud of vapour — was it taking off?

No. Ignition was due at zero seconds. Lift-off at +3s. Four seconds, five seconds after zero — was it going to explode, like the first V2s, or like the previous European rocket, Europa, which blew up shortly after take-off? "Mondieu" said the radio man next to me into his telephone. I peered more closely at the screen, thinking he'd spotted something. But then the vapour drifted away, Ariane looked just as before. Anticlimax. ESA officials, long-faced, made for the bar, where the whisky bottles were quickly emptied.

Then the details began to come through. One of the four main engines of the first stage had failed to reach the planned pressure of 9 atmospheres at its combustion chamber — it had reached only 5 atmos — and the computer automatically controlling the last five minutes of launch wisely decided to close the fuel valves. According to some

accounts this decision had been made within 0.1s of ignition, accounting for the puff of vapour.

When the disappointment had abated a little, Brian Stockwell, deputy head of the Ariane programme in ESA, said that he'd give 50-50 that it was an instrument rather than an engine failure. "The engines have been fully qualified" he said. Engines early in the development phase had failed to reach design pressure, or had done so more slowly than others, but the problems had been ironed out. The particular engines on this vehicle had never been fired, but they came from a series which was fully proven.

By Monday, detailed records made at the Kourou, French Guiana launch site of the operation of the engines were supporting Stockwell's opinion. An ESA statement said that a malfunction of two pressure sensors on one of the engines "is very clearly shown by the recordings and is attributable to a local overpressure occurring in their feed line shortly after engine ignition, thus putting them out of order. Appropriate steps to avoid a recurrence of such an incident are under study".

Re-launch is planned for next week, the statement says, the time being taken up by re-equipping the vehicle with seals that have been broken and reversing other 'one-way' changes that are made in the last few minutes of the launch sequence. Ariane must also be checked to see if its brief ignition caused any damage.

Officials are therefore hopeful that Ariane will fly with the first and second



Ariane just before zero seconds

stages functioning normally. But the third stage, using liquid hydrogen and oxygen, is more doubtful.

While versions of the first and second stages have both been fired successfully in ground tests, the third stage remains to be 'qualified'. In its most successful ground firing, the engines burned only for 90s, instead of the designed 150s. Full qualification is not expected until February, before the launch of a second Ariane; so ESA officials are looking on this first launch as a development flight rather than a test launch. And if of the first four launches planned for the next year, two Arianes successfully put their test packages into orbit, ESA will consider they have a success.

Robert Walgate

UK genetic engineering regulations relax gradually

THE outcome of the UK Genetic Manipulation Advisory Group's programme to assess the risk from recombinant DNA experiments has been a gradual lowering of the categories of physical containment under which many experiments are now being done, according to Sir William Henderson, chairman of the group. The programme, suggested by Dr Sydney Brenner of the MRC Laboratory of Molecular Biology, Cambridge at the end of 1978 (*Nature* 276, 104; 1978), identified the type of host vector system as a crucial factor in designing safe experiments.

The development of safe host vector systems over the past year has meant that about 90% of recombinant DNA experiments can now be done under the first two categories of physical containment laid down by the Williams guidelines. Experiments which still come under categories III and IV are mainly those involving the expression of toxins or hormones.

GMAG had been slightly embarrassed, said Sir William when introducing the group's second annual report last week,

when the National Institutes of Health in the US proposed 'relaxing' its guidelines about a year ago. At that time, genetic engineering experiments in the UK still came under the more stringent guidelines laid out in the Williams report. Now, however, it was extremely difficult to detect the practical difference between the two systems, said Sir William. The percentage of experiments coming under categories I and II had increased steadily over the past few months from 66% in July to 90% in December.

The procedure of notification of experiments has also changed over the past year. Notification to GMAG and the Health and Safety Executive is now compulsory. Synopses of experiments together with details of the DNA and host vector systems to be used have to be submitted to both bodies and local genetic manipulation safety committees have to recommend the category under which they think the work should be done. Work under categories I and II can begin as soon as GMAG and HSE are notified.

Initial concern that local committees

would be incapable of judging categories has not been substantiated. In only one case, said Sir William, did GMAG have to increase the category of containment and on a few occasions it had even recommended lower containment with the use of improved host vector systems.

Despite the success of the past year, however, the members of GMAG still see a need for careful monitoring of recombinant DNA experiments. Most experiments are now done with disabled host vector systems which would not have been developed if GMAG had not existed, said Professor Bob Williamson of St Mary's Hospital Medical School, London and a member of the group. If GMAG ceased to exist now most of the awareness amongst scientists of the need for safety in experiments with recombinant DNA, which it had helped to create, would vanish.

The major problems which GMAG will be tackling over the coming year include industrial scale-up — one case has already arisen — and genetic manipulation in crops.

Judy Redfearn

Poland steps up research into mining safety

POLAND celebrated St Barbara's day, (4 December) — the traditional miners' festival — in the shadow of three major disasters that cost the lives of 66 Silesian miners in October. Not surprisingly, the official celebrations stressed the importance of mining safety. Speaking at Katowice, the mining centre of Silesia, party leader Edward Gierek, himself a former miner, called upon "all Polish scientists . . . to develop research into greater safety in mining". At the same meeting the Minister of Mining, Włodzimierz Lejczak, announced that this year Poland is spending some 15,300 million zloty on mining safety.

Coal accounts for 95% of Poland's energy needs and is a major hard-currency earning export, bringing in some \$1,700 million annually; total production now runs at some 200 million tonnes annually. However, Poland's mines are particularly hazardous. Recently, Dr Jerzy Matuszewski of the Central Mining Institute in Katowice told *Nature* that in virtually all Polish mines there is a considerable methane hazard. Moreover underground mechanisation is increasing the dust hazard, and the great depth of the pits — about 1000m — exacerbates the risk of rock-falls.

Dr Matuszewski is director of the institute's own experimental mine — appropriately named "Barbara" — where a network of galleries (5 km in all), provided a test-site for the study of gas explosions.

Researchers also study dust control at the "Barbara" site. The dust control section produces spectacular demonstrations of the almost instantaneous combustion of methane when mechanical cutters are used without the water spraying now obligatory in mines using such cutters. Less spectacular, but equally important, are the routine tests on the combustibility of conveyor belts, the monitoring of surface movements, the design of protective clothing, and various anti-silicosis measures.

Mining safety research, in fact, extends far beyond those institutes specifically associated with mining. The Institute of Geophysics of the Polish Academy of Sciences, for example, is currently carrying out a geodynamic survey to attempt to predict earthquakes in mining areas. A special fire-extinguisher recently introduced in hard-coal mines was developed in collaboration with the air-force technical institute. And the Department of Electromagnetic Wave Theory of the Technological Institute of the Academy of Sciences have produced a



Experimental explosion: Poland stresses mining research after major pit disasters

system of radiocommunication for mines.

So much for the physical hazards. What about psychological stress? Although there are some psychological experiments going forward — mostly psychomotor studies — little is said officially about the role of psychological stress in mining accidents. And no official statistics of day-to-day accidents are published — although 'unofficial' estimates place the 1978 fatality figure at around 180. One mining official suggested that "production targets" were the greatest single cause of accidents — "but there is little can be done about that!"

Mr Gierek, in his own tribute to the miners' festival, spoke of the new "four-shift" system (three shifts a day, plus one shift entirely off). This was officially introduced two years ago, is being implemented in an increasing number of mines, and is aimed at "improving our miners' working and living conditions". Mr Gierek failed to mention that all three of the mines involved in the October disasters had "four-shift" schedules.

Computers can replace maths for scientists, says Soviet professor

COMPUTERS must inevitably change the whole structure of mathematical training, from scientists down to schoolchildren, according to Professor Aleksandr Kitaigorodskii. Writing in the prestigious Soviet weekly, *Literaturnaya Gazeta* (No. 43, 1979), he envisages a future with a small computer on every classroom desk, where pure mathematics will become a form of art and "culture", and problem solving will have virtually vanished from the academic syllabus. With the advent of the computer

as "co-respondent", says Kitaigorodskii, the long "marriage" of mathematics and science is ripe for "divorce".

His article is clearly intended as something more than idle futurology. A well presented "press campaign" is a standard Soviet gambit for informally airing a proposed major policy change. A few issues later, (no. 49), an article by Professor M Evgrafov of the Mathematical Institute of the Academy of Sciences of the USSR ostensibly presented a counter-argument. This, however, while counselling caution in planning reforms, only served to reinforce the theme of mathematical irrelevance. Evgrafov claimed in particular that "not a single result obtained in pure mathematics" has had any practical applications for at least 50 years, while by that time "90%" of all such results have now been completely forgotten; and even at the time of its discovery, the "most elegant mathematical result" will be understood by "not more than 100 persons". Since, moreover, so few people are capable of "creative mathematical work", he finds the current insistence on mathematical training in higher education and research institutes, "wanton", "rigid", and "useless".

As for problem-solving, he suggests that in the future this will become pre-eminently the domain of the programmer ("a person of middle-level qualifications") and only if the problem has been wrongly formulated will it be necessary to revert to the "tried



An apple for the programmer

and tested" method of the "Old Testament" — calling in a highly qualified mathematician.

More vehement counter-arguments have been reduced so far to small type at the foot of a column. They deal mainly with a computer's inability to "solve" anything, or, more specifically, to deal with continuous or infinitesimal processes.

A somewhat more original suggestion is advanced by A Badaev, a Candidate of Technical Sciences (roughly equivalent to a PhD) from Penza. Without the common language of mathematics, he says, science would soon be reduced to a state resembling "the well known Biblical legend of the Tower of Babel".

Vera Rich

Native peoples oppose uranium mining

Representatives of native peoples met in Denmark recently to discuss the problem of uranium mines on their lands. **Roger Moody** reports

John F. Racmussen.

THE First International Conference on Uranium Mining in the Third and Fourth Worlds was remarkable, not only in its attendance of 500 anti-nuclear activists and representatives from the developing world, but in the fact that it occurred at all. Held in Copenhagen on 25-27 October, the conference was organised at short notice by three Danish anti-nuclear groups.

Taking advantage of the fact that two US Indian delegates, Winona LaDuke and Herb Blatchford were visiting Europe (see *Nature*, 11 October 1979), the Danish anti-nuclear group 'OOA' issued invitations to activists and representatives of native peoples from Africa, Australia and Greenland.

Just three weeks before the conference, the ruling Siumut party in Greenland had strongly condemned the involvement of their economy in the nuclear age precipitated by an energy-hungry EEC. In particular Siumut vetoed further immediate development of the Narssarsuaq uranium deposit (27,000 tonnes) on the far south-western tip of Greenland. And by coincidence, two Australian Aborigines also happened to be visiting Europe at the time of the conference. They were trying to set up an Aboriginal information centre one of whose aims would be to work for a moratorium on the development of the Ranger, Nabarlek and Jabiluka deposits, all on Aboriginal land in the deep Australian north.

Mick Miller of the North Queensland Land Council, the largest single body representing any of Australia's original people, talked about the struggle for land rights, pointing out that Aborigines had been aware of the potentially devastating nature of uranium "thousands of years ago". Using the powerful images of Aborigine culture, Miller told of an Aborigine belief about the land on which the Ranger mine is due to go on stream around 1981. The story has been told through generations that if the earth is disturbed on this spot, *gabo djang* (green ants) will rise through the crust and destroy the world.

Herb Blatchford's forebears had also sensed the enormous importance of the 'Four Corners' area into which they moved and settled 4000 years ago (the Four

The Narssarsuaq seaboard: Greenland has vetoed further development of uranium

Corners comprises New Mexico, Colorado, Utah and Arizona). However, when uranium mining started in New Mexico (initially for US nuclear weapons) about 30 years ago none of the Navajo or Pueblo people in the Four Corners was told the full extent of what was going on: "We asked — why do we feel the nausea, see our hair fall out, why do our teeth weaken, why do we get up in the morning and have no breath? We went to books, looked back at history. Then we went to the Japanese. We went to Enuwetok atoll and asked them. Then we went to the Nevada Platte people. They all gave us the same answer: *Radiation*."

Greenland's delegates seemed unaware of the full implications of allowing uranium mining on their land. Robert Petersen (surely the only Inuit eskimologist in the country) received polite applause when he reflected the Siumut position that mining should only proceed "if all the dangers of radioactive pollution and waste dumping are solved". Of one thing, Petersen was quite certain: "The mine cannot be a solution to the increasing unemployment among our people. If Narssarsuaq goes ahead, it will produce only a few jobs for our people, and bring in even more outsiders."

Another Greenlander took issue with Petersen on this, and became the only delegate to approve of mining "provided the safety problems are solved" since "we must have an economic base on which Home Rule can work". Jorgen Hertling is a member of the Atassut party which has strong links with Denmark, Greenland's former colonial rulers.

The two high spots of the conference were both unexpected. Immediately after Hertling left the platform — to stony silence — two young Greenlanders rose to announce (in both Inuit and English) that they had just decided to form an anti-uranium mining group in their country. During the next few months, they will travel round the scattered seaboard communities mobilising against the Narssarsuaq project, and strengthening the veto already imposed on it. (There is a

widespread fear among Greenlanders that the Danish government will override the veto, under pressure from the EEC.)

But most surprising was the address given by the only 'Third World' delegate, Hadino Hishongwa from Namibia. Hishongwa is the South West Africa Peoples' Organisation (SWAPO) representative in the Nordic countries, Austria and West Germany. Although opposition to foreign control of Rössing, the world's largest operating uranium mine (1978 production: 5000 tonnes uranium oxide), received majority support in the UN Assembly four years ago, SWAPO has never argued against uranium mining as such until now.

"Uranium is an evil", announced Hishongwa. "It is a threat to Namibia and a threat to the entire world. SWAPO doesn't want to exploit it, even when we are independent."

As well as the Rössing mine (in which Britain's Rio Tinto Zinc Corporation has a 46.5% beneficial interest), South Africa's huge General Mining Corporation and France's Aquitaine are involved in prospecting further uranium lodes in the Swakopmund area, and Namibia's actual deposits could rival those in Gabon and Niger.

So ended a conference which was rather short on facts, somewhat higher on energy and certainly bounding with rhetoric. Whether native or 'natural' peoples in the developing world live on 80% (as was claimed by the conference organisers, OOA) or 40% (my own estimate) of the West's reasonably assured uranium reserves, they are certainly the most vulnerable groups. And if uranium exploration and mining expands by up to 150% — as this year's Uranium Institute's annual symposium proposed it should — future conferences should see greater representation from native peoples who did not hear about, or could not get to, the Copenhagen gathering. □

Roger Moody is an editor of Natural Peoples News published by Colonialism and Indigenous Minorities Research/Action

news in brief

Dioxin traces found in soldiers exposed to defoliant: Research workers at the Veteran's Administration in Washington announced last week that tests had revealed the presence of small amounts of dioxin in fat tissues taken from men who were exposed to the defoliant 2,4,5-T during the Vietnam war. Dr Lyndon E Lee told a public meeting of the VA's Advisory Committee on Health-related Effects of Herbicides that analysis had revealed traces of dioxin ranging from three to 57 parts per trillion in fat tissue taken from 33 individuals, including a control group of 10 who had not served in Vietnam. However he added that the amounts of dioxin, a highly toxic contaminant, were so small that it was impossible to attach any significance to them.

Meanwhile President Carter has announced the setting up of an interagency working group to study the long-term health effects of exposure to Agent Orange and other herbicides which were used extensively during the Vietnam war, and which many veterans are now claiming responsible for complaints ranging from liver disorders to chromosomal damage. According to the White House the working group will have the major responsibility for making public the results and implications of research on the health effects of phenoxy herbicides and their contaminants.

London School of Hygiene and Tropical Medicine fears closure:

London University's School of Hygiene and Tropical Medicine may close if the government's proposed increase in fees to foreign students goes ahead. The school now charges overseas students £1200 per year which would have to increase to £5000 next year and to £7000 by 1982-1983 if the government goes ahead with its plan. Of the school's 249 full time students and 1023 part time students 58% are from overseas from a total of 85 countries. The school is a postgraduate institution offering MPhil, MSc and PhD degrees and is known for research and teaching in tropical diseases as well as community medicine, occupational medicine and epidemiology and statistics. Gordon Smith, dean of the school, said that the probable loss of overseas students was not just a question of money. "Our students are a resource of considerable magnitude. They are experienced and knowledgeable and the school's research programmes could not function without them." Smith also stressed that since the Soviet Union was the only other country offering research and training in tropical medicine at a "reasonable fee" that a policy directed against overseas students might have "undesirable foreign policy implications".

Other internationally known schools of London University threatened by closure because of the proposed increases include the School of Oriental and African Studies with 35% overseas students and the London School of Economics with 37% overseas students.

ASTMS calls for MRC tenure reforms: The Association of Scientific, Technical and Managerial Staffs is discussing proposals for extensive reforms in the UK Medical Research Council's tenure procedures. A working paper circulating to ASTMS/MRC national advisory committee affirms ASTMS general policy that "potential dismissal, including the end of fixed term contracts, should be the subject of negotiation and not be solely at the employer's discretion". The proposals call for changes in both new appointments and tenure procedures. Present non-renewable three year appointments would be replaced by three year fellowships with a one year renewable option, a retirement contribution, no age limit, no waiver clause and would end with a "substantial cash gratuity if the fellow is not taken on to permanent staff". The proposed tenure procedures for scientific staff would replace the present five year scheme with a one year probationary period followed by a tenure decision. The tenure decision would be made with the candidate having full access to submitted reports and in the case of denial reasons would have to be given.

UK National Physical Laboratory Chemical Division to close: A 4% cut in the budget of the National Physical Laboratory is to be applied solely to the division of Chemical Standards. Chemists in the division were told that a decision had been "made centrally" to close two sections in the division because "the jam had become too thin to spread further". The two sections, Reference Materials and Thermodynamic and Phase Equilibria Data, employ two thirds of the Division's 60 scientists and the entire division will close as a result. The scientific staff has been given two years and three months to find other jobs. It is not clear whether the NPL will employ them since the Chemical Standards Division is the only one devoted to chemistry at the NPL.

UK energy report calls for development of fast breeders: A major Department of Energy report on energy technologies for the UK regards the fast breeder reactor as the only economic solution to Britain's electricity needs over the next fifty years. On the basis of the assumptions made in the report it is estimated that it will ultimately be economic to install nuclear power to generate between 80% and 90% of public electricity supply. The fast breeder is seen to be the likely solution for the ensuing world-wide competition for uranium. The report says "Only by introducing the fast reactor on a large scale by the early decades of the next century can the world demand for uranium be brought within credible bounds". Fusion power is seen to be unrealistic and alternative sources of energy are regarded only as insurance against shortages. *Energy Technology for the UK* Vol 1 (£3.00) Vol 2 (£5.75). HMSO.

UK to provide free science consultancies for industry: A scheme to facilitate "industrial exploitation of innovations originating in research" has been approved by the Science Research Council and the Royal Society. The two bodies will provide £100,000 a year for ten fellowships to enable academic scientists to "work directly in the productive process" and for industrial scientists to learn new basic research techniques. The scheme is expected to be effective in "growth technology areas" in which there is "quite a distinct demand on the industrial side". Although the scheme is intended to be "flexible" genetic manipulation techniques are expected to be in high demand in industry circles.

BSSRS calls for new safety procedures for hospital radiation hazards: The British Society for Social Responsibility in Science Hospital Hazards Group has issued a report calling for new radiation limits and redundancy agreements for hospital workers exposed to radiation. The report suggests that trade unions should negotiate local limits at each hospital that are substantially lower than established upper limits since "all research indicates that the majority of exposure can easily be kept at 1/10 of the current limits".

The report also calls for trade unions to negotiate lay-off agreements whereby workers who receive their maximum allowable yearly dose of 5 rem are transferred to new jobs without loss of pay or seniority. In addition the report recommends that protective safety clothing be a measure of "last resort". Substitution (e.g. ultra sound for x-rays) and containment or shielding are seen to be superior safety measures. *Radiation Hazards in Hospitals*. British Society for Social Responsibility in Science, 9 Poland St, London W1. 18p.

Correction: In the correspondence "Fast reactors will be problematical and expensive" (22 November, page 358) there was an error in the fourth sentence of the second column. It should have read "... spokesmen for the Nuclear Installations Inspectorate stated "we have indicated the seriousness with which the NII views the possibility of whole-core accidents in fast reactors. The consequences are potentially worse than for major accidents in a thermal reactor system ...".

Recombinant DNA: have recent experiments assessed all the risks?

Experiments to test the safety of cloning viral genomes in *E. coli*, published earlier this year, have allayed fears about the risks of recombinant DNA. But, argue **Barbara Rosenberg** and **Lee Simon**, the experiments actually indicated some worrying aspects of the behaviour of recombinant DNA.

Two reports on the assessment of risk from recombinant DNA were published in March, 1979 in *Science* (203, 883; and 887). These experiments were designed "to evaluate certain postulated mechanisms of possible biohazard stemming from recombinant DNA research with *E. coli*". The authors "view these results as being highly reassuring with respect to the safety of cloning viral genomes in *E. coli*". This statement, in our opinion, reaches far beyond the results presented. The new data actually confirm several aspects of hazard mechanisms that have been postulated, but they do not address other aspects that must be investigated before the overall hazard can be evaluated. Nevertheless, the published conclusion has been widely accepted and the reports have been cited as cause for the relaxation of fears about risk to the public of gene-splicing experiments (for example *The New York Times* 2 May; *Nature* 15 February, page 505).

A team headed by Wallace Rowe and Malcolm Martin of the National Institute of Allergy and Infectious Diseases did the research at Fort Detrick. Their experiments tested the ability of certain recombinant molecules containing polyoma virus DNA to cause viral infection in mice (polyoma virus grows readily in mice and is easily detected). Subsequently, a more cautious report of very similar experiments, sponsored by the European Molecular Biology Organisation (EMBO) appeared in *Nature* (28 June, page 811). The EMBO group prepared the same types of recombinants and tested for virus production in cultured mouse cells; they plan to test later for infectivity in animals. The assay used in both studies detected infectious virus particles produced by the recombinant molecules, and so required total functioning of the recombinant polyoma virus DNA to produce a positive result.

Only a relatively uncommon type of recombinant molecule was found to produce infectious particles. (This contained a tandem polyoma insert, which because of its head-to-tail double structure is capable of regenerating free circular polyoma DNA within the cell.) Ordinary recombinant molecules with a single polyoma insert did not do so. This is indeed expected in the light of current molecular biological knowledge. To produce infectious virus from a single polyoma insert would require specific mechanisms for excision or replication which are not known to exist.

Furthermore the particular recombin-

ants chosen for study are incapable of the full genetic function necessary for virus production. They were all constructed with enzymes that cleave polyoma DNA within a transcriptional region and in the resulting recombinants a part of the genetic information is disjoined from its control region and could only be reconstructed by regenerating free circular polyoma DNA. The production of free virus particles by recombinants with a single polyoma insert would thus require a highly unlikely intracellular event. The observed lack of infectivity conveys a false sense of security which arises from the method chosen to evaluate the possible biohazard.

In fact the production of complete, infectious DNA viruses by recombinant DNA, as tested in these experiments is one of the least probable hazards of recombinant DNA technology. There are other dangers in cloning viral DNA that are at least as serious, and far more probable: in particular the possibility that one or more viral genes could function without the production of a complete virus. In the case of tumour viruses such as polyoma, partial genetic functioning of the virus DNA is considerably more dangerous than the production of infectious virus. The action of a single viral gene can transform a cell towards malignancy and consequent tumour formation, whereas complete virus function leading to a productive infection is harmless to mice.

The functioning of single genes from other sources, for example toxin-producing genes, could also be hazardous. This is the kind of risk posed, in general, by the cloning of eukaryotic DNA (DNA from organisms other than bacteria). Single gene function is a 'worst case' hypothesis — it suffices, for example, for a scenario which envisages the spread of microbial-borne cancer. A test for single gene function in recombinant molecules therefore provides a much better basis than infectivity for conclusions on the safety of the technology.

The team led by Rowe and Martin have now published information on this subject (*Science* 205, 1140; 1979). Their results show that polyoma virus DNA in recombinant molecules induce tumours in newborn hamsters with a frequency similar to that of non-recombined polyoma DNA; the recombinant molecules can therefore function genetically in mammalian cells. These positive results indicate that recombinant DNA can be highly hazardous if it is brought into appropriate contact with an animal.

In these tumour induction experiments,

19% of animals injected with intact, uncleaved polyoma DNA developed tumours, and 19% of animals injected with intact tandem-insert polyoma DNA also developed tumours; intact recombinant λ or plasmid DNAs with single polyoma inserts gave variously 0.6 and 9% tumorigenicity (Israel *et al.* *Science* 205, 1140; 1979; Table 2). The overall variation in the tumorigenicity of intact recombinant molecules, 0-19%, is a result of the small numbers of animals seen; similar variability was seen in similar conditions for tumorigenesis by free intact polyoma DNA (*J. Virol.* 29, 990; 1979; Table 5).

An accurate, quantitative determination of tumorigenicity is not possible from the data given, but it is clear that for both single and tandem-insert recombinants the tumorigenicity is of the same order of magnitude as that of free polyoma DNA. One can conclude that the insertion of polyoma viral DNA into a vector molecule from a different source does not significantly change its tumorigenicity and is, therefore, not a safeguard. Moreover, recombinant DNA can clearly enter hamster cells and the region of the inserted DNA required for tumour induction evidently finds genetic expression. This indicates the potential genetic activity of recombinant molecules containing DNA from any source, not just viruses.

The successful infection experiments with recombinants containing tandem polyoma inserts also indicate that recombinant DNA can enter mouse cells. The authors point out that the recombinant DNA is not recognised there as foreign and that the polyoma DNA retains its biological activity after cloning in bacteria. These experiments also reveal a new element in the hazard that may be posed by genetically active recombinant molecules. The recombinant molecules became 100-1000 times more infective when they were enclosed in λ -phage particles (*Science* 203, 887; Tables 2 and 3), although it is not emphasised in the text. The phage coat apparently protects the DNA from degradation and facilitates its entry into mouse cells. Thus, polyoma- λ recombinants, both single and tandem are endowed with a large advantage over free polyoma DNA. A close comparison of the polyoma dosages in free DNA and λ -phage particles in the tumour-induction experiments (*Science* 205, 1140; Table 2)

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confirms this advantage. Thus, if *Escherichia coli* containing polyoma- λ recombinants should come into contact with appropriate animal cells, λ particles possessing recombinant DNA molecules could readily enter the cells and act genetically to induce tumours.

The key question now is whether *E. coli* (or other hosts) containing recombinant DNA can provide a new route by which viral or other genes could find their way to sensitive species.

This question is not addressed in those risk-assessment experiments undertaken so far, although it must be answered before we can be reassured as to the safety of cloning recombinant viral DNA in *E. coli*. Although one of the papers from Rowe and Martin's laboratory states that *E. coli* containing polyoma-recombinants were not infective when injected into mice, this is misleading because they tested too small a dose of the recombinant contained in *E. coli*. In the case of free recombinant DNA a larger dose is required (their Table 2 and 3). (The same is true in the tumour-induction experiments.)

E. coli containing polyoma recombinant DNA induced no tumours (*Science* **205**, 1140; 1979; Table 1) but for technical reasons the polyoma dose contained there in was an order of magnitude lower than the minimum dose required for measurable

results with the number of animals used, as shown by the DNA data (Table 2). Moreover the authors point out that experiments involving injection of live *E. coli* may tend to be biased in favour of negative results.

Since most of the animal data were obtained by injection, the relative infectivities or tumorigenicities obtained in this way reflect the ability of the DNA molecules (either free or in phage particles or bacteria), to enter and act in mouse cells with which they have artificially been placed in contact. But injection is not a normal mode of accidental exposure of an animal to bacteria.

There are many normal ways in which *E. coli* can accidentally enter mammals and proliferate at various sites; urinary tract infection or gut colonisation are typical examples. If an *E. coli* strain containing a recombinant were able to colonise an animal in these or other ways, or transfer the recombinant to another bacterium that could do so, the amount of the recombinant could eventually be greatly amplified internally. The animal would then be exposed to the recombinant continuously over a long period of time, thereby increasing the risk substantially, even if the event is of low probability when infection by polyoma virus itself is considered. Via the recombinant, polyoma

genes would be given a new route of access to the animal.

E. coli host-vector systems have been developed with the aim of minimising this possibility, but their reliability has not yet been demonstrated. Indeed, risk-assessment experiments in progress under contract to the US National Institutes of Health indicate that survival of EK2 strains in the human gut (other sites have not been systematically tested) and in the environment is considerably greater than originally anticipated, and plasmid vectors can increase survival. The likelihood that recombinant EK2 strains may be able to transfer genetic information to indigenous microorganisms in various natural environments may therefore be significant, but this has not yet been adequately tested.

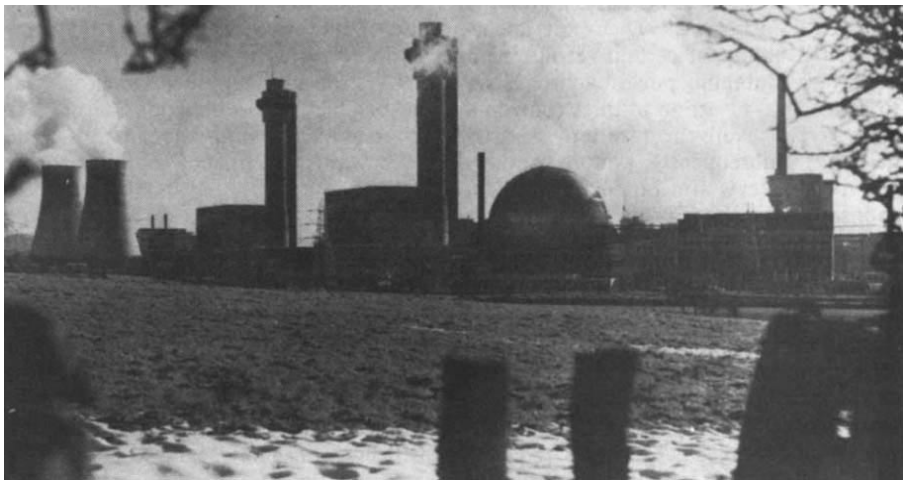
The extent of the hazard posed by a potentially functional recombinant clearly depends on whether the host-vector system used can provide a new route to the animal, or plant, or to man. The main import of the present risk-assessment experiments which in effect rule out any automatic safeguards at the levels of cellular entry and gene function, is to give new importance to the question of new routes of genetic access that could be provided by *E. coli* or other cloning hosts. Until this has been fully studied, there is no basis for reassurance on the risks of recombinant DNA. □

Nuclear power: the threat to personal freedom

Robin Grove White looks at the implications for civil liberties in the UK of a major increase in nuclear power.

The UK government's proposal to expand the nation's nuclear capacity will increase debate on the long term impact of nuclear power on civil liberties. This issue has already become central to the debate in other European countries.

The Royal Commission on Environmental Pollution first raised the issue of nuclear power and civil liberties in its sixth report (Cmnd 6618) in 1976. Together with subsequent independent studies, the report argued that a major commitment to nuclear power, and in particular to the plutonium fuel cycle, would require extensive security measures to safeguard nuclear materials such as plutonium from theft and sabotage. These safeguards would damage relations between the



individual and the state.

Extensive vetting of workers inside and outside the electricity industry, the growth of unconventional armed protection for nuclear facilities and materials, wide-ranging surveillance of dissident groups and constraints on the flow of information about nuclear power to the news media would all contribute to increasing authoritarianism.

The government and the nuclear industry were not impressed with these arguments. In June 1977, the Department of Energy said that future nuclear plant could be designed to eliminate many of the potential civil liberties' hazards. Industry spokesmen such as Sir John Hill Chairman of the Atomic Energy Authority (UKAEA)

went further: a combination of technical fixes such as radioactive 'spiking' of fuel elements, siting fuel fabrication plants and power stations in the same place and physical obstacles at vulnerable points in the fuel cycle would obviate the need to intrude on civil liberties. In any event, it was maintained, thieves were unlikely to be interested in materials such as plutonium.

Such confidence is increasingly hard to endorse. No such technical fix has been found to be politically or economically feasible and there have been ominous developments.

On 26 October 1979, the first International Convention on the Physical Protection of Nuclear Materials (for peaceful purposes) was concluded in

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Vienna. The convention established draconian measures to protect the use, transport and storage of civil nuclear materials, including the exchange of information between national security authorities, rigorous vetting of individuals with access to sensitive points in the fuel cycle, arming of guards and an agreement that all exchanges of information concerning nuclear security should be confidential. The convention is far franker in its recognition of the hazards of civil nuclear power than the UK.

The security community has recently said that surveillance, vetting and secrecy will play a growing role in economies opting for nuclear power. The 1978 Rand Corporation Report, *Attributes of Potential Criminal Adversaries of US Nuclear Programs*, stressed the limitations of physical security in guarding against theft or sabotage of civil nuclear materials. Rand lays emphasis instead on the need for more stringent security clearance procedures, for secret armed police and for a pervasive air of 'uncertainty' (i.e. secrecy) to thwart nuclear hijacks.

This agrees with a senior police official who recently said at a high-level UK seminar, that, for nuclear materials, investment in intelligence tends to be more cost-effective than an equivalent investment in physical containment.

An indicator of the growing gap between the UK's earlier official reassurance and

"not more than a few hundred" extra armed constables would be required to guard even this massive programme. However, between 1976 and 1979, the constabulary's strength increased 50%, from 400 to 600 — and at a time when there have been no additions to the civil nuclear programme.

There have also been developments in the levels of vetting now thought appropriate for staff of relatively low skill in the nuclear fuel industry. There has always been extensive vetting of the personal associations and political affiliations of employees of the UKAEA and British Nuclear Fuels Ltd. The extent of such security screening has now become a significant irritant in union-management relations. At a conference on Trade Unions and Nuclear Power in November 1978, a senior official of the Electrical, Electronic, Telecommunications and Plumbing Union (himself no critic of nuclear expansion) expressed dismay at the escalation of security vetting at Windscale.

Recent foreign experience suggests that intensified surveillance of bodies opposed to nuclear power is a likely concomitant of nuclear expansion. Reports from West Germany, Australia and the United States point to cases of spying and infiltration of campaigning groups. Over the past few months in the UK, commentators as various as the *Daily Telegraph*, Sir Fred Hoyle and the Institute for the Study of

his 1978 report on the Windscale inquiry, Mr Justice Parker could offer "no solution at all". He opted for security and silence.

In June 1977, the Energy Secretary, Tony Benn, stressed that "the government are well aware of the need for discussion of such an important issue to take place on an informed basis . . . [They] will make every effort to ensure that adequate information is available. They are confident that it will be possible for Parliament to perform its traditional function in this area". Yet just six months later, in reply to a Commons question about the range of security measures needed for fast breeder reactors, Mr Benn was brutally laconic: "I can only give the classic answer: that I am advised that the increase in surveillance and vetting will not go above a normal level".

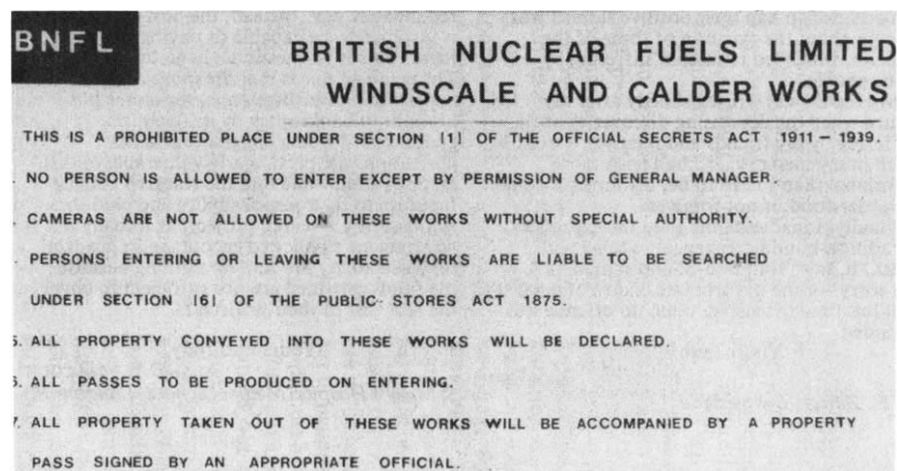
Powerful arguments for greater openness have been made. In a recent article in *International Relations*, Cambridge historian Christopher Andrew urges: "As the dreadful day of the terrorist with a nuclear device in his suitcase draws gradually and inevitably closer, intelligence surveillance of potential terrorist groups will have to be increased. And it is essential that increased surveillance should have the support of informed public opinion . . . The only alternative to a public informed about the intelligence services is a public misinformed about and suspicious of them".

But is such openness credible, or even desirable? Each additional nuclear commitment compounds the tensions over civil liberties as it leads towards ultimate reliance on the plutonium-fueled fast breeder reactor. In this way, argue the civil libertarians, new thermal nuclear power stations are contributing towards new and unhappier relations between the individual and the state. In April 1977, for example, the International Commission of Jurists, a group of leading lawyers concerned with human rights and the law, met in Vienna and expressed "grave concern about the dangers to human rights created by nuclear weapons and materials and nuclear power programmes". It called on all governments to "take these dangers into account before forming a policy on nuclear power".

Tony Benn was undoubtedly worried about these issues when he was Energy Secretary, as some of his public statements testify. But there is little in the public record to suggest that civil liberties have weighed significantly in the decisions of David Howell, his successor.

It is conceivable that the pursuit of nuclear power will damage civil liberties less than would the pursuit of an alternative long term energy strategy, such as the low energy growth programme outlined earlier this year by the International Institute for Environment and Development. But the government has certainly not demonstrated this.

The most important social dimension of nuclear technology may be going by default in Whitehall. □



hard reality is the growth since 1976 of the UKAEA's Special Constabulary. This private armed police force operates on a constitutionally unique basis, its actions shrouded from public (and parliamentary) view for security reasons. Justice, an all-party association of UK lawyers, has commented that the constabulary's structure "conflicts with all our traditions of civilian and politically accountable policing".

In February 1977, Friends of the Earth and others asked the then Energy Secretary, Tony Benn, to comment on the likely scale of the constabulary's expansion to meet the security demands of a hypothetical 127GW nuclear programme. His department's considered reply was that

Conflict have pointed to the potential advantages to the Eastern Bloc of a strong UK anti-nuclear movement. It is likely that such views are already reflected in UK security, given the problems that could arise from disruption of the nation's energy supply and the dangers of nuclear materials in the wrong hands.

But how far will such surveillance extend? And how is it to be kept within bounds? Two rival imperatives clash. Public understanding of the full implications of nuclear power demands openness about civil liberties' before irrevocable decisions are taken. But the imperative of security (itself the very source of the difficulty) dictates silence.

Weighing evidence about this conflict in

correspondence

Stench on the New Jersey Turnpike

SIR, — The central point in Thomas Jukes' recent review of *Malignant Neglect* (8 November, page 166) — that the book treats facts cavalierly — is amply documented by the quotations he cites. I should like to point out, however, that Professor Jukes' own attitude to facts is not beyond criticism.

Jukes quotes the book's description of the "stench" and emissions from the large petrochemical complex near the New Jersey Turnpike, which (it implies) is related to 14,000 cancer deaths in New Jersey. He then dismisses the statements on the grounds that he once drove the same route at 6.45 pm and observed neither stench nor emissions, apart from steam.

Having myself travelled this route, by car or train, at least 50 times over the past 20 years, I can assure Professor Jukes that the petrochemical stink in the area is no man's invention. Since I did not keep score, I cannot swear to how often I experienced it — but it was often enough for the experience to become an absolutely expectable feature of the trip.

Jukes' own account provides a clear explanation of why he smelt nothing — assuming, of course, that the complex was in operation at 6.45 pm. "To the west," he notes, "there was a brilliantly clear sunset in which a range of hills, 30 miles away, was sharply etched against the sky." That degree of visibility could exist only amid a continental polar air mass entering the area — which is normally associated with northwesterly winds. The complex lies southeast of the highway.

New Jersey does in fact have one of the highest cancer rates in the US. Whether this has any connection with emissions from the chemical manufacturing facilities with which northern New Jersey is sown has yet to be established. However, a number of reputable authorities, including the US Public Health Service, consider the possibility real enough to be worth investigating.

Yours faithfully,

ROBERT CLAIBORNE

New York, US

Research ceased during the Cultural Revolution

SIR, — Having recently returned from working at the Institute of Vertebrate Palaeontology and Palaeoanthropology in Peking I would like to challenge Tilman Spengler's assertion (15 November, page 224) that this was one of the institutes of the Chinese Academy of Sciences that "worked very well" during the Cultural Revolution. Certainly the institute was not physically closed down, but a research institute where no research is permitted, can hardly be said to be working well. By 1972 research activity was starting up again, but for six years nothing was heard of vertebrate palaeontologists or their work. The Chinese vertebrate palaeontology journal *Vertebrata Palasiatica* ceased publication in 1966 and did not reappear until 1973. The impression gained from my Chinese colleagues was that the Cultural Revolution was a period of enforced isolation and stagnation.

Finally, I would like to comment upon the

statement attributed to the three Chinese scientists visiting Britain: 'In China we tend to work more collectively. It is unheard of for an individual to work alone . . .'. I suspect this comment may well have arisen from a semantic misunderstanding. Certainly from my observations, Chinese scientists in my own field, at least, work exactly as they do in Britain. The individual scientist is employed with others in an institute, will collect raw data jointly with colleagues, will discuss his or her results with colleagues, may cooperate with others to produce joint papers, but in the main does scientific research alone. This much is evident from even a cursory glance at the authorship of Chinese scientific publications.

Yours faithfully,

L.B. HALSTEAD

University of Reading, UK

China has many aspects

SIR, — In your introduction to the two reports on "China: the legacy of the Cultural Revolution" (15 November, page 224), you used the phrase "views of China are as diverse as its visitors", and seemed to imply a disagreement between myself and Tilman Spengler. I would like to point out, however, that the diversity is a reflection of the "diverse aspects of the problem", and not necessarily a "diversity of opinions". Spengler discussed policies, which had their positive sides; I was talking about the execution of some of the policies, which led to intense suffering by some people.

My report was written shortly after my return when the depressing discoveries of the suffering by my friends and relatives were still fresh in my memory. If I had been more emotional than I need to be, my motives could be understood, if not forgiven.

Finally, I inadvertently used the expression "tradition-bound arrogance" when I really meant to say "tradition-bound smugness". I am sorry for the unfortunate choice of a word that has been offensive, when no offence was intended.

Yours faithfully,

KEN HSU

ETH, Zürich, Switzerland

New select committee seeks expertise

SIR, — The Education, Science and Arts Committee held their first meeting on Thursday, 29 November.

In recommending the setting up of the new select committees, the Procedure Committee stated, "We hope that, as the new committees become established . . . they will develop methods of work which will enable them to respond more speedily to current problems and to new policy proposals". In view of this we have decided to study a number of subjects and report fairly rapidly during the coming year. By fairly rapidly we mean we are trying to set ourselves a maximum time of two months between starting to take evidence and reporting. We have facilities to hire specialist assistance. We have not yet decided which shall be our first subjects of study but we expect them to be in the field of education.

The Committee are anxious to ascertain what specialist assistance might be available to them in their investigations, and I have therefore written to a number of institutions asking if and how they are able to help. But the committee would also like to hear from any individuals and other organisations who feel they have the individuals and other organisations who feel they have the necessary expertise, and I am therefore seeking the hospitality of your columns to make this request.

Yours faithfully,

CHRISTOPHER PRICE MP

House of Commons, London UK

Research councils: do they pay the real cost?

SIR, — I should like to publicise an unfortunate consequence of the restrictions which grant-awarding bodies such as the Science Research Council place on the use to which their grants may be put. In general the recipient is not allowed to use the money to pay for computing, telephoning, typing, photocopying, page charges for publishing, library facilities, recruitment advertising, removal expenses for staff appointed, or redundancy pay. Instead, the host institution is assumed to be capable of paying for all of these. This is unquestionably no longer true. The result of this is that the more active a department or institution is, the closer it is brought to bankruptcy by its scientific endeavour. I would suggest that small institutions are particularly vulnerable to this. Is it not about time that the research council faced up to their responsibility and paid the full cost of a research project? Is it really our government's policy to encourage its academic scientists to lay low and do nothing because the funds provided are not sufficient to cover the real cost of their projects?

Yours faithfully,

ALAN D.B. MALCOLM

St Mary's Hospital Medical School, London UK

X as the Y of Z

SIR, — In your review of Horace Judson's *The Eighth Day of Creation* (11 October, page 506) I am called "a sort of Louella Parsons of molecular biology" apparently, because I told Judson that I thought a friend of mine is its Douanier Rousseau (a painter whom I very much admire). Here your reviewer is not far off the mark: long ago I picked up the habit of styling X as the Y of Z at a party in Hollywood, where I heard Christopher Isherwood say that Wagner is the Puccini of music.

Before finally kicking the habit, may I coin one final simile? Namely, if *The Eighth Day of Creation* really showed as much lack of historical comprehension as your reviewer found it to show, then Judson would be the Thomas Jukes of molecular biology.

Yours faithfully,

GUNTHER S. STENT

University of California, Berkeley, US.

news and views

Mutants in a mosaic gene reveal functions for introns

from Bernard Dujon

Two years ago molecular geneticists discovered an unexpected organisation for eukaryotic genes: the DNA sequences that specify the primary structure of the gene product (often called exons¹) are interrupted by other base sequences (called introns or intervening sequences). The origin and the significance of the latter remain a puzzle (see ref. 2). However, one might hypothesise that the different alternating pieces of the same gene, the introns and the exons, could perform different genetic functions recognisable with the help of specific mutations. A mitochondrial gene of the baker's yeast seems to provide an example of this.

After mutations in the mitochondrial gene for cytochrome b, an enzyme of the respiratory chain, were first reported about three years ago³⁻⁵, it soon became apparent that they result in a wide diversity of phenotypes^{6, 7}. Some of them are even pleiotropic and also block the synthesis of the subunit 1 of cytochrome oxidase, another element of the respiratory chain whose gene is in another part of the mitochondrial genome. By a combination of genetic and molecular methods^{8-11, 16} and by a laudable collaborative effort between different groups of investigators, all the mutants (around 200) are now ordered in a unique map¹².

The idea that the cytochrome b gene is a mosaic¹³ of, at least, five exons interspersed with four introns is suggested by the following lines of evidence. (1) There is a consensus that the gene product is the 30,000 molecular weight polypeptide, from specific labelling of the mitochondrial translation products^{10, 14-16}. However, mutations affecting cytochrome b span a region of the mitochondrial DNA which is several times larger than needed to code for this polypeptide and are highly clustered (refs 9, 11, 12 and Fig. 1). (2) The smallest transcript found in this region of the map (2.3 kilobases) and believed to be the mature messenger RNA for the cytochrome b²⁰⁻²², hybridises with non-contiguous segments of DNA²⁰ whose positions and sizes are consistent with expectations from the genetic and physical

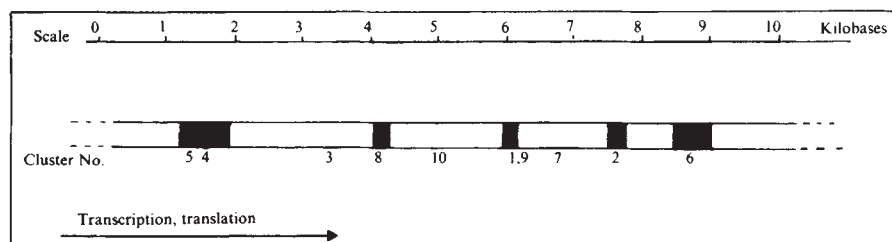


Fig. 1 The cytochrome b gene is a mosaic of at least five exons (black) interspersed with four introns (white). Mutants can be found in both (cluster No.). They have been mapped as indicated by a combination of genetic and molecular methods.

map. (3) With some mutants the 30,000 molecular weight polypeptide is replaced by a single new polypeptide usually smaller than the wild type^{14, 16, 18} as if premature chain termination had occurred. Colinearity¹³ between the size of the new polypeptide synthesised and the location of the mutants on the map strongly suggests a single translation unit. This is confirmed in double mutants by an epistatic effect of the upstream mutation on the downstream one^{18, 19}. (4) With other mutants, interspersed among the first ones, a series of new polypeptides appears in the range of about 15,000 to 60,000 molecular weight^{14, 16, 18}, suggesting that translation now proceeds also through sequences not normally translated. Peptide mapping confirms that for most cases the new polypeptides do share sequence homologies with the wild type protein^{16, 17}.

A unique contribution of the yeast cytochrome b gene to the more general question of the expression and regulation of eukaryotic split genes lies in one's ability to perform functional complementation tests between mutants. On the basis of this test the mutants fall into two classes^{9, 19}. The first is composed of mutants that fail to complement one another (they belong to the clusters 5, 4, 8, 9, 1, 2 and 6; numbering is historical and not logical). Those therefore are satisfactorily explained by the classical notion of cistron and they are called here 'exon mutants'. The idea that they are mutants in exons is confirmed by the fact that drug resistant mutations, affecting the sensitivity of the protein to specific inhibitors but not its activity, are closely linked to them^{23, 24}. Now, most 'exon mutants' can be complemented *trans* by the mutants of the second class

(clusters 3, 10 and 7). Thus, mutants of the second class, although interspersed among those of the first, do not belong to the same cistron. Instead, each cluster constitutes a new complementation group. In a mosaic gene the classical notion of the cistron must therefore be accommodated with the fact that some mutations in the continuity of a given cistron will not belong to that cistron, as if different mutants affected different types of functions. Mutants of the second class are described in the literature as 'intron mutants'. Such a definition only refers to the functions altered in the mutants. The location of these mutants within long introns can eventually be established by molecular mapping^{9, 11}. These mutants therefore suggest a specific role for introns. Interestingly, they show the pleiotropic effect on cytochrome oxidase.

The next refinement in complementation studies was achieved by experiments in which the exons of the two parents are distinguished from each other by the use of the drug resistant mutations as genetic markers. From a series of such experiments performed recently²⁵ it became clear that the 'exon mutants' can provide in *trans* a function that helps overcome the deficiency(ies) of the 'intron mutants', such that the final gene product can be made using the non-mutated exon sequences of the 'intron mutants'. Consistent with the fact that 'exon mutants' are a unique cistron, all the exons are now always expressed in *cis*. This is diagrammatically shown in Fig. 2. Similarly different 'intron mutants', belonging to two different clusters, can help each other such that both will now be able to express normally their non-mutated exon sequences.

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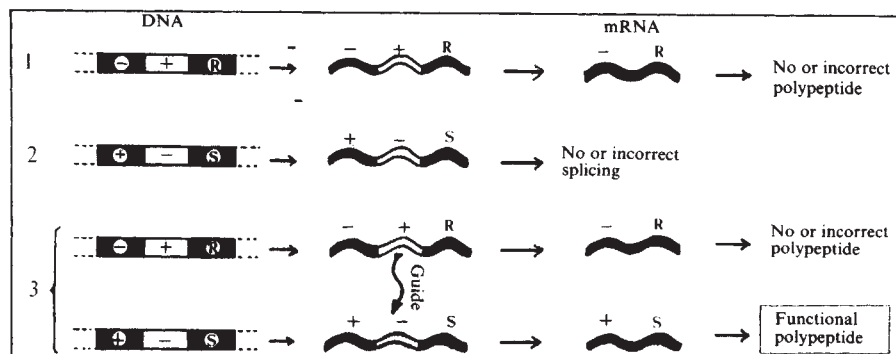


Fig. 2 During complementation (3) a wild type 'guide' function from the exon mutant (1) helps overcome the splicing deficiency of an intron mutant (2) whose non-mutated exon sequences are then used to direct the synthesis of the polypeptide. The 'guide' step is arbitrarily shown as an interaction between two mRNA precursors but could be otherwise.

All the properties of 'intron mutants' reflect a deficiency in a gene function, different from the coding of the primary sequence of the protein, and required at an earlier step than translation. From this we hypothesise a deficiency of RNA splicing. This is directly confirmed by examination of the RNA transcripts showing that 'intron mutants' are actually blocked at different specific steps of splicing^{21,22}. Correct RNA splicing therefore would require wild type sequences which, when mutated in a given gene, can be complemented *in trans* by products of another copy of the same gene. This prompted P. Slonimski to propose a new element for gene expression called 'guide RNA'^{21,26}, whose role is to align the splice junctions or to pull out the skein of messenger RNA precursor for proper splicing to occur. The guide concept is based on the *in vivo* complementation test and is necessary to explain the results. The idea that such a guide role is played by an RNA molecule, and not a protein, is not yet directly demonstrated. But it opens the possibility that successive processing of the different introns could serve as a mechanism for regulating gene expression. Such schemes are now being proposed^{21,22}.

In those the completion of one step requires the success of a previous step, as if proper splicing of one intron created the guide needed for the splicing of another. The same scheme applies to explain the pleiotropic effect of some 'intron mutants' on the cytochrome oxidase gene²¹.

If such a guide exists one would like to

know the nature of the sequences involved, their relations to introns, exons or junctions and the molecular form of the guide (for example, is it free spliced intron RNA or part of the precursor). Similarly the mechanism of guidance (for example RNA/RNA base pairing or more complex interactions) is a question for investigation. In addition, the interaction of some mutants of the cytochrome oxidase gene on the expression of cytochrome b^{21,27} may also be a very promising avenue for study. One can then reasonably guess that in the near future an ordered regulatory scheme of splicing of these two genes, assigning specific functions to specific sequences, will be available. Now, what in the genome of mitochondria reflects a high degree of specialisation and what a more general pattern for eukaryotic genes, remains as yet unknown. But, in any case, the cytochrome b gene immediately provides functions for introns that are directly amenable to experimental analysis. □

Flight formations in geese and other birds

from Robert M. May

AMONG the autumn sights around Princeton are the V-shaped formations of Canada geese, *Branta canadensis*, honking across the sky in flights of up to 100 birds.

It has often been suggested that the purpose of this tidy formation is to take advantage of aerodynamic efficiencies which can accrue to group flight. Figure 1 (from Lissaman & Shollenberger, *Science* **168**, 1003; 1970) illustrates how these effects come about. As these authors explain, the downdraft under the bird (the downward pointing arrows) derives from the "basic principle of aerodynamics that an object flying by dynamic lift in a fluid gets its lift by creating downward momentum within its span. This applies to fish or fowl, airplane or submarine." Beyond the wing there is an upwash, which is very intense near the wingtip and which is associated with conservation of momentum in the fluid. The resulting streamlines are as depicted in the left half of Fig. 1; these streamlines show the direction of airflow generated by the passage of the bird, and we see they have a general tendency to produce an updraft to the right and left of the bird, and a downdraft above and below it. Thus birds flying side-by-side can take advantage of the updrafts from neighbours, an effect similar to flying in an upcurrent, with a consequent reduction in the 'lift power' needed. Conversely, birds flying in a vertical column would each need to

generate additional lift to compensate for the downdraft effect.

In a quantitative analysis of the magnitude of these effects, Lissaman and Shollenberger have shown that a formation of around 25 or more birds can reduce the power required for 'lift' by a factor of 2.9 by flying wingtip-to-wingtip in a horizontal line. For a given power output, the increase in range scales as the square root of this lift-reduction factor; thus flight formations of 25 or so birds can have a range roughly 70% greater than that of a lone bird. The associated optimum cruising speed decreases as the inverse fourth root of the lift-reduction factor, corresponding to a 24% reduction in the speed of the formation compared with a lone bird. These calculations take into account the effects of lift (or, in aeronautical jargon, 'induced drag') and of the power needed to overcome direct air resistance ('profile drag'), but they ignore the complications of flapping wings. The flapping losses are likely to be negligible so long as the wingtip flapping speed is significantly less than the

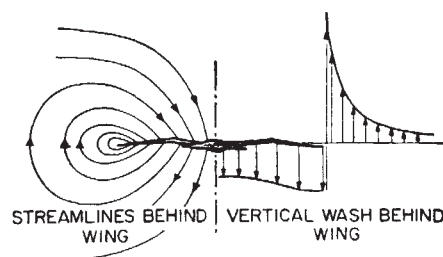


Fig. 1 Flow field associated with a flying bird.

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forward flight speed; this is so for geese, but is not always true for the passerines discussed below.

The basic calculation of Lissaman and Shollenberger is for a horizontal formation of birds advancing in line abreast. But 'Munk's stagger theorem' states that in a classical fluid (where circulation is conserved) any member of an assembly of lifting surfaces may be translated in the direction of its flight path without changing the total induced drag. Now, in the line abreast formation the end birds enjoy relatively less, and the centre birds relatively more, of the upwash; indeed, with more than ten birds, the central birds have double the savings of the end ones. By staggering the formation into a V, the induced drag can be shared evenly, without affecting the total for the formation. The V does this by virtue of the lead bird experiencing a weaker upwash field even though it is mainly on one side. In detail, the 'optimum' configuration is like a slightly rounded V, with a blunted point and more acutely trailing tips. For a formation of nine birds, Lissaman and Shollenberger's calculations suggest the angle of the V to be roughly 120°. The formation has, moreover, a kind of stability in that a bird forging ahead will tend (under constant power) to fall back, and a bird dropping back will tend (under constant power) to catch up; more subtly, the formation is not stable against a bird which drops back and reduces power.

Gould and Heppner (*Auk* 91, 494; 1974) have attempted to test these ideas, by analysing films showing formations of Canada geese in North America. They made observations of more than 100 flights of flocks, averaging around 27 geese. The first disconcerting observation was that over 40% of the formations were echelons, wherein the lead bird must enjoy significantly less than the average updraft. Moreover, in the five cases where the data yielded a steady result, they found (after correcting for the distortions created by viewing the formation from the ground and at an oblique angle) an average V-angle of 35°. Gould and Heppner also find other V-angles of 31° and 36° mentioned in the literature, by people who chanced to see flocks directly overhead. They also report unpublished radar observations by Williams, Klonowski and Berkeley, which give an average V-angle of 71°. (The radar data were collected in the spring, the others in the autumn, and in all cases the geese were flying from feeding to roosting grounds, rather than being in long-distance migration.) In short, almost half the formations were echelons, and those that were V-formations had angles consistently much less than Lissaman and Shollenberger predicted on the basis of equalisation of uplift among the birds.

Gould and Heppner conclude that aerodynamic effects are not the main reason for V-formations, and suggest other explanations (such as the birds keeping



Canada Geese flying

each other in view). I think this conclusion is too strong. Another piece of the information elegantly gathered by Gould and Heppner is the spacing of birds along the arms of the V, which ranges from 3.2 m to 4.7 m for different V-angles. The key distance, however, is this spacing projected along the line of flight (that is, the equivalent line-abreast spacing), which averages about 1 — 1.5 m, or around the wingtip-to-wingtip distance (approximately 1.5 m) that gives maximum overall lift. This arguably is the main phenomenon, and its total magnitude is (according to Munk's stagger theorem) independent of whether the flock flies in line abreast, in echelon, or in a V of arbitrary angle. The question of equalising the advantage among the birds is a much subtler one, and evokes echoes of similar questions about the "geometry of the selfish herd" (Hamilton, *J. theor. Biol.* 31, 295; 1971), where aggregation does confer some overall benefits on the herd, but where at any one moment these benefits are enjoyed more by the inner than by the outer members. It would be interesting to know if the disadvantaged lead goose keeps the lead for long periods, or whether the lead continually shifts.

The preceding discussion pertains to horizontal line formations, as are found in geese. But many other species of birds conduct their long twice-yearly migrations in large three-dimensional flocks. This is the case for most migratory passerines.

Higdon and Corrsin (*Am. Nat.* 112, 727; 1978) have analysed the net aerodynamic advantage associated with such flock-flying, as a function of the overall shape of the flock. If the average separation between birds is reasonably large compared to the wingspan of an individual bird, an elegant nineteenth-century-tripostyle calculation is possible, regarding each bird as a dipole vortex and summing over all birds. For a two-dimensional flock of such birds, randomly arranged within a vertical ellipse of height B and width A , the flock-associated decrease in induced drag (or upwash) on each bird, expressed as a ratio to the induced drag in sole flight is

$$(\pi^3/64)nd^2(A-B)/(A+B). \quad (1)$$

Here n is the average number of birds per unit area and d is the wingspan; thus nd^2 can be rewritten as $(d/\Delta)^2$, where Δ is the average spacing between birds. (The expression (1) differs from Higdon and Corrsin's by an unimportant numerical factor of 4, which they lose in the process of making a sequence of unnecessary approximations.) The maximum aerodynamic advantage is obtained when the horizontal extension of the flock greatly exceeds the vertical ($A \gg B$), whereas if the vertical dimension exceeds the horizontal one ($B \gg A$), the net effect is an increased downdraft. This general observation does not depend on the detailed shape of the flock, and it follows broadly from the earlier discussion

associated with Fig. 1. We can see that, unless the birds fly closer together than observations suggest, the aerodynamic effects of such flight in two-dimensional flocks is slight; even if the spacing between birds is as little as twice the wingspan ($\Delta \sim 2d$), the uplift effect in a horizontally extended flock is only around 10%. Higdon and Corrsin estimate the corrections to equation (1), in the event of the birds flying in some ordered lattice pattern, rather than more-or-less randomly. These correction terms are of order n^2d^4 ; that is, they are smaller than those given in equation (1), by a factor of order $(d/\Delta)^2$.

Extending the analysis to encompass a fully three-dimensional flock is in principle straightforward (by virtue, again, of Munk's stagger theorem, which enables the three-dimensional problem to be projected into a two-dimensional one). In a randomly arranged flock, the upshot will be to regain the expression (1), but with n now being the average number of birds projected onto unit area perpendicular to the flight direction. Clearly, the result will be to increase the uplift effect (1) as n increases, provided the flock is extended more horizontally than vertically. If the effective value of n becomes too large (so that nd^2 is not appreciably less than unity), the 'dipole vortex' approximation will begin to break down, and the computations must be based on pairs of vortices centred at a bird's two wingtips. But for most three-dimensional flocks of migratory birds the density is unlikely to make this refinement necessary. I take home the message that three-dimensional flocks of passerines and other birds can gain non-negligible updraft effects, provided the flock is extended more horizontally than vertically, but that the magnitude of the effect is not nearly as large as that gained in the close, horizontal line formation of geese.

A different kind of insight into the dynamics and ergonomics of migratory flight comes from Allerstam (*J. theor. Biol.* **79**, 341; 1979). Starting with the observation that wind speed is usually less at low than at high altitude, he examines the idea that migratory birds may save flying time and energy by allowing themselves partly to be drifted by strong winds at high altitude, and then correcting their course at low altitude in relatively weaker winds. For typical values of wind velocities, Allerstam's calculations suggest this behaviour will be advantageous if the strong upper winds are blowing in a direction roughly 30° to 90° wide of the goal direction ('following side winds'). If the wind is blowing at an angle less than 30° to the desired direction, a direct high altitude flight is optimal. Conversely, a direct low altitude flight is the best route against a headwind (in excess of 90° wide of the goal direction). Allerstam concludes with a survey of the available evidence, of which the best (Gruys-Casimir, *Arch.*

Neerl. Zool. **16**, 175; 1965) is on chaffinch migration in Holland: "Radar observations of drift in high altitude bird migration and visual records of low altitude overcompensation are compatible with the optimal flight behaviour of migrants at high and low altitude, respectively, as predicted from this hypothesis." □

Mycorrhizal associations

from Peter D. Moore

MYCORRHIZAL associations between plant roots and fungi are now known to be extremely common in nature. It is evident that such associations are symbiotic, both parties benefitting from the interaction. As far as the plant is concerned, the association may be very costly in terms of the loss of fixed carbon to its heterotrophic partner. Lewis and Harley (*New Phytol.* **64**, 256; 1965) fed ^{14}C sucrose to cut roots of beech (*Fagus sylvatica*) and observed that 50–75% of the carbon reaching the root tip was passed on to the mycorrhizal fungus, which obviously represents a considerable drain on the photosynthetic resources of the plant.

The widespread occurrence of mycorrhizae, however, witnesses to the evolutionary value of the partnership to host plant as well as fungus. It has long been established that a major advantage to the plant is the greater efficiency with which it is able to absorb necessary inorganic materials from the soil (Harley *J. Ecol.* **59**, 653; 1971). There is also some evidence to suggest that water uptake is assisted by mycorrhizal infection (Safir *et al. Plant Physiol.* **49**, 700; 1972). This being so, one might expect mycorrhizal associations to be of particular value, and therefore especially prevalent, in habitats deficient in plant nutrients and water.

Grime (*Plant Strategies and Vegetation Processes*, 36; Wiley, Chichester, New York, 1979) takes up this argument in his discussion of stress-tolerant plant species. Grime proposes an ecological classification of plants into competitors, stress-tolerators and ruderals, and he claims that mycorrhizal associations, particularly of an ectotrophic type, are to be expected amongst plant species adapted to nutrient stresses. Data recently published deriving from arid regions of the United States support and extend this view.

Reeves, Wagner, Moorman and Kiel (*Am. J. Bot.* **66**, 6; 1979) have conducted a survey of vesicular-arbuscular (endo-) mycorrhizae in semi-arid sagebrush communities of Colorado. Of the 42 species of plant present, only 2 were found

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to lack mycorrhizal infection, one chenopod and one crucifer. This emphasises the widespread nature of mycorrhizae in a taxonomically diverse flora: 13 families are represented and all but one have mycorrhizal members present.

The authors also investigated a nearby site which had been severely disturbed by road construction. Here 21 plant species were growing, but only 6 possessed mycorrhizal infections; these species had also been present in the undisturbed site. The remaining 15 species were confined to the region of disturbance and had no detectable mycorrhizal infection (three individuals of each species were examined). Thus, in an unstable habitat, the mycorrhizal plants could be at a disadvantage, perhaps because of the destruction of a large proportion of the residual fungus in the soil during the process of disturbance which might restrict recolonisation by mycorrhizal species of plants. The authors propose that early successional stages are deficient in mycorrhizal species, whereas in later, more stable stages the vast majority of species are so adapted. This seems to be justified for their arid, nutrient-poor habitat.

The lack of mycorrhizal species in disturbed sites is confirmed by the results of a similar investigation by Miller (*Can. J. Bot.* **57**, 619; 1979) of mine spoil heaps and nearby undisturbed sage scrub in Wyoming. In these latter, pristine areas, 21 species were examined and only one lacked vesicular-arbuscular mycorrhizal infection. On the spoil heaps 16 species were found, none of which was infected. Even within one family, the Chenopodiaceae, some members are non-mycorrhizal and ruderal in habit, whereas others (the majority) are mycorrhizal and are found in undisturbed habitats where edaphic stresses of nutrient and water availability are encountered.

So the Grime hypothesis seems to hold true for these semi-arid ecosystems: there does appear to be a division among plants which corresponds to his stress-tolerant and ruderal types. What is not clear from these particular sites is the outcome of low disturbance and low stress. In other words, of what importance are endomycorrhizae to the Grime competitor? □

100 years ago

M.A. Guyard claims to have discovered another new metal of the platinum group which he names uradium, from the Ural Mountains, whence the ore is procured. There have been quite a flood of similar announcements lately. We have now gallium, davyum, mosandrum, neptunium, decipium, phillipium, norvegium, scandium, ytterbium, holmium, "X", thulium, and uradium. Chemists will have to keep as narrow a watch on these minor elements as our astronomers do upon the minor planets, or we shall not know where we are.

From *Nature* **21**, 25 Dec., 187; 1879.

The transforming genes of SV40 and polyoma

from Peter Rigby

SIMIAN virus 40 (SV40) and its relative polyoma (which naturally infects mice) are small DNA viruses which have relatively simple genomes that provide attractive and easily manipulated systems for probing the mechanisms of eukaryotic gene expression. They have also attracted great interest because of their ability to transform some mammalian cells *in vitro*. Such transformed cells have many of the properties of tumour cells and have been studied in the hope of elucidating the mechanisms by which viral gene expression can over-ride the normal controls on cell growth and behaviour. During the past few years great progress has been made in defining the viral genes and their products responsible for transformation. It has been known for some time that the so-called 'early' region (those genes expressed soon after infection and before viral DNA replication in cells in which the virus is able to multiply) is sufficient to induce transformation. Work on the transforming capacity of SV40 and polyoma has therefore focused on defining the products of the early region, and recently published work, together with results presented at two meetings* last summer, have greatly increased our understanding of the structure of this region and of the properties of its protein products.

The availability of complete DNA sequences for both SV40 and polyoma¹⁻⁸ has enabled the relationship between the various protein products of the early region, classically identified by immunoprecipitation with sera from animals bearing virus-induced tumours, to be clarified. Originally only one such SV40 'tumour' antigen (T-antigen) was recognised. This is a protein of apparent molecular weight 100,000, now called large T-antigen; a protein of this size would account for almost all the coding capacity of the early region. Prives *et al.*⁹ then showed that among the products of *in vitro* translation of viral mRNA obtained from infected cells was another immunoprecipitable protein of molecular weight 17,000 — the small t-antigen.

Large T/small t

The first clue to the origin of small t came with the discovery of viable SV40 deletion mutants that produce normal large T despite the fact that the deletions are within the sequence that was thought to code for this protein¹⁰. However, these mutants do not induce the synthesis of full size small t¹¹ and transform cells at a reduced frequency under certain assay conditions^{12,13}. At about the same time Berk and Sharp¹⁴ showed that there were two SV40 early mRNAs and not one as had previously

been thought. These mRNAs have the same 5' and 3' ends but are spliced differently (see Fig. 1). The smaller of the two early mRNAs encodes large T¹⁵; the sequences missing in the viable early deletion mutants correspond to those normally spliced out of this mRNA and so the protein is unaffected. In the other mRNA fewer nucleotides are removed by splicing and the additional mRNA sequences contain an in-phase termination codon. Thus this message directs the synthesis of small t¹⁵. The relationship between the two tumour antigens shown in Fig. 1 has been confirmed by peptide mapping and sequencing studies¹⁶⁻¹⁹.

The analogous polyoma mutants, known as *hr-t* (host range-transformation) mutants, were isolated many years ago by Benjamin²⁰ and are absolutely defective for transformation. *hr-t* mutations, which can be either deletions or point mutations, map in the region of the polyoma genome analogous to that deleted in the viable early deletion mutants of SV40^{21,22} and it was shown that polyoma also encodes a small t which is affected by *hr-t* mutations although the large T remains normal^{23,24}.

Polyoma middle T-antigen

Immunoprecipitation of extracts of polyoma-infected and transformed cells with anti-tumour serum has identified a third tumour antigen, of molecular weight 55,000 — middle T-antigen²⁵. This is found in association with the plasma membrane and probably has the same amino-terminal sequence as small t and large T²⁶⁻²⁸. But peptide maps show that middle T contains peptides found in neither of the other two tumour antigens suggesting either that it is translated in a different reading frame or that it is partially coded by the host cell^{26,27}. However, the mRNA for middle T hybridises specifically to polyoma DNA²⁹, this protein as well as small t is altered by *hr-t* deletions^{24,28} and examination of the polyoma DNA sequence reveals the presence of a second open reading frame in the appropriate part of the early region.

SV40-transformed cells contain an immunoprecipitable protein of similar molecular weight, 53,000, and it was initially thought that this might be the analogue of polyoma middle T. The relevant region of the SV40 genome, however, contains only one open reading frame, that used for large T, and it is now clear that this protein, which is discussed in more detail below, is of cellular origin.

Organisation of integrated viral DNA

In cells transformed by SV40 or polyoma, viral DNA is covalently integrated into the chromosomal DNA of the host cell and the expression of this viral information is responsible for the transformed phenotype. There is a great deal of new

information on the organisation and expression of these integrated sequences which is helping to identify the roles of the different tumour antigens in transformation. The organisation of the integrated viral DNA in many transformed mouse and rat cell lines has been analysed by restriction mapping. The general conclusion is that integration is not specific, each line has a characteristic pattern of virus-specific DNA fragments indicating that many different sites on both viral and cellular DNAs can be used for integrative recombination³⁰⁻³³. However, F. Cuzin (Nice) has reported that identical integration patterns are observed in two sets of SV40-transformed cells, one set of three rat lines the other of two mouse lines. There are distinct biological differences between the three rat lines but in view of the preponderance of data suggesting random integration the significance of these observations is unclear.

The structure of the integrated viral DNA can provide clues to possible mechanisms of integration. A high proportion of SV40 and polyoma-transformed cell lines carry tandem duplications of all or part of the viral genome^{32,33}. Such structures would not be expected if recombination occurs between monomeric, circular viral DNA and linear chromosomal DNA. However, P. Rigby (Imperial College, London) discussed data indicating that SV40 DNA replicates in mouse cells to produce large, tandemly-arrayed polymers of viral DNA and that these polymers recombine effectively with one another. If such polymers are the precursors of the integrated form the high frequency of tandemly-duplicated insertions would be explained. When some transformed cells are fused with permissive cells infectious virus can be rescued and in certain cases the DNA of the rescued virus is identical in structure to that of the virus used to transform the cells. Such perfect excision may well result from the presence of tandemly-duplicated insertions and the loss of a complete copy of the viral genome from a tandem array has been demonstrated in some polyoma-transformed lines³⁴. However, excision does not require tandem duplication; when the transformed rat cell line 14B, which carries a defective copy of the viral genome, is fused with permissive cells imperfect excision occurs giving rise to a heterogeneous population of circular molecules containing both viral and cellular sequences (M. Botchan, Cold Spring Harbor Laboratory, New York). Some of these rescued DNAs have been cloned and sequence analysis shows that the structure of the viral-cellular joins is unaltered. It has been proposed that this type of excision may proceed by way of *in situ* replication of the integrated viral

*XLIV Cold Spring Harbor Symposium on Quantitative Biology 'Viral Oncogenes', 30 May-6 June 1979; and the ICRF 1979 Tumour Virus Meeting, 16-20 July, 1979, Cambridge.

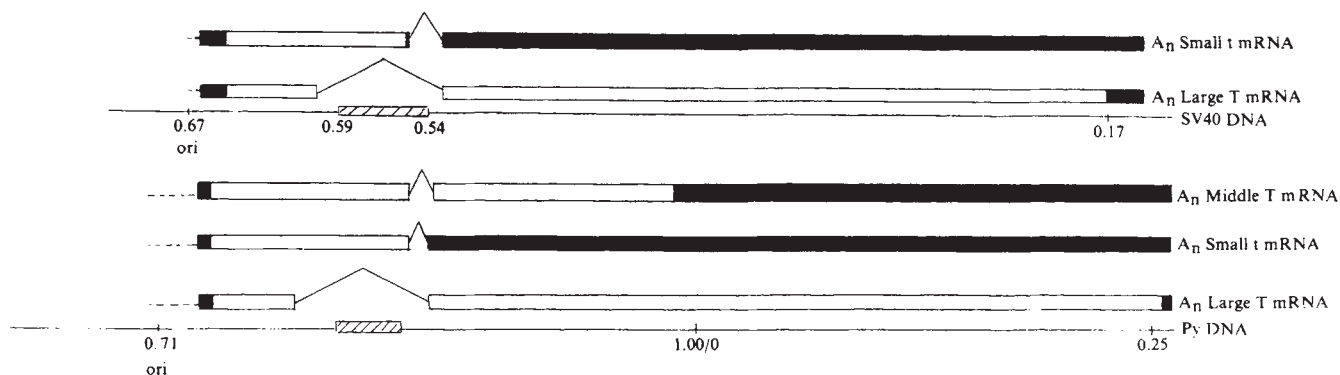


Fig. 1 mRNAs found in cells productively infected by SV40 or polyoma. ■, Non-translated regions; □, translated regions; △, splices; ▨, regions of DNA deleted in viable early deletion mutants (SV40) or *hr*-mutants (Py); ---, 5'-end heterogeneities.

sequences rather than by a recombinational excision³⁵.

J. Sambrook and J. Stringer (Cold Spring Harbor Laboratory, New York) described the cloning of the single integrated SV40 genomes of two transformed rat cell lines. In one line, 14B, the integrated sequences are not colinear with free viral DNA; one segment of the genome has been deleted while another is inverted, giving a structure that Sambrook suggested could be derived from recombination between cellular DNA and a replicative intermediate of viral DNA. The cellular-viral DNA junctions in this line have been sequenced; there is no detectable homology between the viral DNA and the cellular DNA into which it is integrated suggesting that integration occurs by illegitimate recombination. The cellular sequences flanking the integrated viral DNA have been compared in detail to those flanking another integrated viral genome that has been cloned, from line 17, and there are no detectable similarities or sequence homology.

T antigens and transformation

Although there is much evidence to implicate large T in transformation by SV40, elucidation of the structure of the integrated viral DNA in polyoma-transformed cell lines suggests that polyoma large T is not essential for transformation. Some polyoma-transformed cell lines do not carry complete copies of the early region; although such lines may have tandemly duplicated insertions each copy of the early region is interrupted, either by a deletion or by a virus-host junction, in such a way that there is no complete coding sequence for large T (M. Fried & L. Lania, ICRF, London). Analyses of the mRNAs (R. Kamen, ICRF) and of the tumour antigens present in such lines confirm that they contain small t and middle T but not large T, although in some lines truncated derivatives of large T are present.

More direct evidence that polyoma transformation does not necessarily require intact large T is provided by experiments in which viral DNA, either intact or cleaved by restriction endonucleases, is injected into newborn

hamsters³⁶. If the DNA is cleaved to interrupt the early region coding sequences unique to large T, tumorigenicity is increased over that of supercoiled intact DNA and cell lines established from the resultant tumours contain only small t and middle T. It might be argued that large T is produced from uncleaved or recircularised DNA during the initial stages of tumour induction. This possibility has been eliminated by experiments in which hamsters are injected with a recombinant plasmid containing polyoma DNA cloned through its *EcoRI* site, which lies within the unique large T coding sequence, into the *EcoRI* site of pBR322 (M. Martin & M. Israel, National Institutes of Health, Bethesda). Tumours were obtained and cell lines established from them. Such lines lack large T and analysis of the DNA of one of them shows that the integrated DNA retains the two polyoma-pBR322 joints. J. Hassell (McGill University, Montreal) reported data showing that cloned fragments of polyoma DNA that contain the coding sequences of small t and middle T, but lack almost all the sequences unique to large T, will transform cells *in vitro* and that the resulting cell lines have a standard transformed phenotype. Further confirmation of the role of the various tumour antigens in polyoma transformation is provided by an experiment in which rat cells were infected with an *hr-t* mutant and cell lines were established from clones picked at random³⁷. One such line contains the genome of the *hr-t* mutant in both free and integrated forms. The cells synthesise large T but not small t nor middle T; they have a normal phenotype *in vitro* and will not induce tumours *in vivo*, showing that large T itself is insufficient to maintain the transformed state. Mutants of polyoma with deletions in the region coding for both middle T and large T have altered transformation properties³⁸ and some of the cell lines that are isolated do not have all the characteristics of a fully transformed cell (B. Griffin, ICRF). When taken together these data imply that only half of the early region is required to initiate and maintain transformation and suggest that middle T may be the most important protein for this process.

Temperature-sensitive polyoma mutants of the *ts-a* class map in the region coding uniquely for large T^{39,40} and it was shown many years ago that, at the non-permissive temperature, such mutants are defective for the initiation but not for the maintenance of transformation⁴¹. It may well be that in a normal virus infection large T is required to initiate transformation, perhaps because integration requires viral DNA replication, as has been suggested for SV40. Transformation with DNA involves the cells receiving massive doses and in such cases other integration mechanisms, not dependent on DNA replication, may operate. What is clear is that polyoma-transformed cells can retain their phenotype in the absence of intact large T, whereas all SV40-transformed lines examined so far retain and express all of the large T coding sequences. The difference between the two viruses presumably reflects the fact that polyoma has evolved a third early protein, middle T, which SV40 does not have.

Polyoma transcription

The transcription of the early region of polyoma DNA has been studied in great detail by Kamen and his colleagues. Their powerful two-dimensional modification of the Berk and Sharp S1 endonuclease mapping procedures reveals three major mRNAs in productively-infected cells (see Fig. 1) which presumably code for the three tumour antigens. The mRNAs for small t and large T have a similar relationship in both polyoma and SV40. Fitting the S1 mapping data to the DNA sequence indicates that the mRNA for middle T is derived by splicing the leader of small t mRNA to a body that begins some twelve nucleotides downstream of the body of small t mRNA, thus eliminating the termination codon used for small t and effecting the change in reading frame necessary to produce middle T. Polyoma-infected cells also contain a minor class of mRNAs, containing representatives of all three splicing patterns, the 3' ends of which map close to the middle of the early region, just past the termination codon for middle T.

In polyoma-transformed rat and mouse cells the 5' ends of the mRNAs correspond

to those found early in productively-infected cells, suggesting that transcription is initiated at the viral rather than a host cell promoter. Almost all transformed cell lines overproduce the shortened mRNAs that have polyadenylated 3' ends mapping in the middle of the early region and in one mouse line the vast majority of the viral mRNA terminates at this site, although the virus-host junction in the integrated DNA is 0.75 kilobases further downstream. There has been much speculation regarding the existence in transformed cells of fused virus-host transcripts. The integrated viral DNA from one mouse line, *ts-a3T3-3*, has been cloned in λ (Fried) and Kamen's group have mapped the mRNAs against this isolated DNA segment. Their data show clearly that the polyadenylated ends lie some 60 nucleotides into the cellular DNA at the 3' end of the transcription unit, thus providing the first definitive evidence for fused transcripts.

SV40 transcription

Transcription in SV40-transformed cells has not yet been analysed in the same detail. Sambrook reported that the mRNAs of the rat lines 14B and 17 had been mapped against the cloned integrated genomes using the S1 endonuclease method; they seem to be identical to the two early transcripts found in

productively-infected cells. However, M. Lovett (Imperial College, London) presented transfer hybridisation experiments⁴² showing that in several mouse lines novel transcripts, both larger and smaller than those found in infected cells, can be detected. One line contains derivatives of both small t and large T mRNAs that extend past the normal viral polyadenylation signal but it is not clear whether they terminate at a viral or a cellular site. E. May (IRSC, Villejuif) discussed S1 mapping data suggesting that some rat lines synthesise mRNAs which contain tandem duplications of viral sequences coding for large T. *In vitro* translation of such transcripts shows that they encode very large proteins related to large T; these super T-antigens are quite widespread⁴³⁻⁴⁵. Peptide mapping indicates that in some cases super T proteins contain peptides from the C-terminal half of large T in more than one molar yield (F. Chaudry, ICRF) suggesting that the amino acid sequence is also tandemly duplicated.

A cellular protein associated with T-antigens in SV40-transformed cells

As well as the virus-coded T-antigens SV40-transformed cells contain an immunoprecipitable protein of molecular weight 53,000, the so-called nvT-antigen (non-viral T-antigen). Peptide analysis shows that this protein is unrelated to small t or large T and that it is different in transformed cells of different species⁴³⁻⁴⁵. It has also been found in uninfected mouse 3T3 cells and undifferentiated mouse teratocarcinoma cells^{46,47}. Its synthesis is induced by SV40 infection and this induction requires the activity of large T⁴⁷. In extracts of transformed cells nvT cosediments with the polymeric, highly-phosphorylated form of large T (F. McCormick, ICRF). These experiments confirm the proposition, first advanced by Lane and Crawford on the basis of immunological experiments⁴⁸, that nvT is precipitated by anti-tumour serum because it exists intracellularly as a complex with large T. G. Jay and E. Appella (NIH) reported that a 53,000 molecular weight protein, which may be identical to nvT, is recognised by antisera raised against methylcholanthrene-induced mouse sarcomas and that this protein is expressed in cells transformed by many agents including SV40. It is found in chemically-induced sarcomas, leukaemias induced by either X-irradiation or Moloney murine Leukaemia virus, in cells transformed by the Moloney or Kirsten strains of murine sarcoma virus and in spontaneously-transformed fibroblasts. The protein is also detectable in undifferentiated F-9 teratocarcinoma cells but not in normal 3T3 cells, primary embryo cells, adult lung fibroblasts, haematopoietic cells or lymphoid cells. Most interestingly the protein is present in normal thymus cells which have an extremely high mitotic rate and it appears to be synthesised only at

particular stages of the cell cycle. It is tempting to speculate that nvT is involved in the control of cellular DNA replication and that complex formation between it and large T is part of the mechanism by which the virus over-rides the normal control of cellular DNA replication.

T antigens as enzymes

The enzymatic function(s) of the papovavirus T-antigens continues to be somewhat controversial. Following the demonstration that the transforming protein of the RNA tumour virus Rous sarcoma virus (RSV) has protein kinase activity⁴⁹ several groups reported that SV40 large T has a similar activity^{50,51}. However, R. Tjian (University of California, Berkeley), working with the hybrid protein encoded by the adenovirus-SV40 hybrid D2, now claims that the kinase activity can be separated from the D2 T-antigen by purification procedures which do not separate the ATPase activity also associated with this protein. Glycerol gradient centrifugation experiments show that the ATPase binds well to SV40 DNA, a property expected of an activity of large T which binds to the viral origin of DNA replication^{52,53}, whereas the kinase binds rather poorly. Tjian has now isolated large T from SV40-infected monkey cells; although it has not been purified as extensively as the D2 protein, 99.9% of the kinase activity can be separated from the large T is two chromatographic steps. Moreover, large T isolated from cells infected with *tsA* mutants has a thermolabile ATPase activity but the removed kinase is thermostable. L. Hager (University of Illinois, Urbana) also reported work indicating that large T has an ATPase activity that is stimulated by oligo(dT) but that it is not a kinase.

However, D. Livingston (Harvard University) presented data indicating that highly purified preparations of large T from transformed human cells do have a kinase activity and that the protein isolated from cells infected with *tsA* mutants is temperature-sensitive for the transfer of phosphate to casein. The situation remains confused but it seems that the identification of SV40 large T as a protein kinase is somewhat suspect. It may be unreasonable to expect the enzymatic activities of the transforming proteins of SV40 and RSV to be the same, particularly as large T is predominantly nuclear whereas the *src* protein is located on the plasma membrane⁵⁴.

None of the polyoma tumour antigens has been purified but there are suggestions that middle T, which is found in the plasma membrane, is at least associated with a kinase activity (W. Eckhart, Salk Institute; B. Schaffhausen, Harvard University; A. Smith, ICRF). This work relies on a kinase assay in which phosphate is transferred

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from ATP to immunoglobulin or to middle T itself in immunoprecipitates of polyoma tumour antigens; it is thus impossible directly to implicate a virus-coded protein in the enzymatic reaction. However, kinase activity is detected in plasma membrane fractions, the location of middle T, and in transformed cell lines which lack large T but contain middle T. Moreover, there is no kinase activity in the cell line that contains the integrated DNA of an *hr-t* mutant and thus produces large T but not middle T. These results raise the possibility that middle T is a kinase, but they cannot rule out that the enzyme activity is a property of one of the other, less well characterised, viral proteins found in immunoprecipitates, or of a cellular protein that binds specifically to middle T.

In the immediate future we can expect the application of recombinant DNA techniques to lead to a detailed description of the integrated viral DNA found in transformed cells and of the mRNA species synthesised from it. In the longer term the central question to be addressed regarding the molecular biology of SV40 and polyoma will concern the enzymatic activities of the tumour antigens and the cellular targets on which these proteins act.

Ancient plate speeds

from Peter J. Smith

ABSOLUTE speeds of the Earth's lithospheric plates are generally lower for those plates having the greater proportions of continents, higher for those plates having the lengthier subducting edges, and higher for those plates with the greater colatitudes. But can these generalisations be regarded as universal rules holding over all time, or are they merely conclusions temporarily valid under current circumstances? Certainly the statistics are hardly impressive enough to inspire confidence in universality. It is true, for example, that all the plates having large continental components ($>2 \times 10^7$ km²) have low speeds (< 30 mm a year); but there are only four of them, or not really enough to support the conclusion that large continents always move slowly. More generally, it would be rash to impute timelessness to any conclusion drawn from the behaviour of the mere 10-12 major plates in existence today.

On the other hand, investigation of past plate speeds is difficult. One approach would be to calculate absolute plate motions on the assumption that the Earth's hot spots are fixed with respect to the mantle; but it is not entirely clear that the assumption is valid, and ancient hot spot

traces are difficult to recognise anyway. Solomon *et al.* (*J. geophys. Res.* **82**, 203; 1977) made rather more progress when they estimated plate motions during the early Tertiary on the assumption that there was no net torque between the lithosphere and mesosphere, concluding that generalisations derived from current plate behaviour were not always true in the past. But there is a limit on how far back in time this method can be applied, for it requires complete knowledge of ancient plate boundaries. It is also rather sensitive to the (usually unknown) amount of intraplate deformation which has occurred since.

In view of such difficulties, Gordon *et al.* (*J. geophys. Res.* **84**, 5480; 1979) have now devised a third method of estimating ancient plate motions — one based on palaeomagnetic data. The advantages of such a technique are that palaeomagnetic results for many continents cover at least several hundred million years and that it is possible in principle to determine apparent polar wandering paths with considerable accuracy. The most serious disadvantage, however, is that palaeomagnetism does not give the information necessary to specify continental motion in full. What Gordon and his colleagues have done is to devise a method whereby the data inherent in the polar wandering curve are converted into the rms velocity of the plate — a by no means simple procedure, for the precise relationship of apparent polar wandering to continental drift depends on the position of the continent with respect to the plate's pole of rotation. Because of the fundamental incompleteness of palaeomagnetic data, however, what emerges is not the actual rms velocity of a plate but a minimum rms velocity.

Obviously this is not ideal, but it turns out to be far from useless. Gordon *et al.* have applied their technique to the polar wandering curves for North America, Eurasia and Gondwanaland as specified by Irving (*Nature* **270**, 304; 1977) for the past 350 million years. The results clearly show that the relevant minimum rms continental velocities (V_m) have certainly not remained constant over that period. For North America V_m peaked about 190 million years ago at a value of more than twice the present one, and similar maxima occurred for Gondwanaland a few tens of millions of years earlier and for Eurasia slightly later. Indeed, the peak values of V_m were almost as great as the actual rms velocities of present oceanic plates. In each case the period of particularly rapid motion probably lasted 20-30 million years, which is more than sufficient to indicate that plates with large continents can move rapidly when circumstances permit.

In other words, both largely oceanic and largely continental plates are capable of moving rapidly (that is, at velocities greater than 60 mm a year) — which must mean that the resistance to motion of continental lithosphere cannot be substantially greater than that of oceanic lithosphere. The

implication is that the present low velocities of continental plates arise from an accident of plate geometry. Gordon and his coworkers go on to speculate that the chief factor governing plate velocity is the proportion of plate boundary attached to subducting slabs. It just so happens that at present the largely continental plates are short on subduction zones; *ergo*, they are now moving only slowly.

Nevertheless, it may be quite wrong to conclude from all this that at any given time continental plates are just as likely to be travelling as fast as oceanic plates. Continental plates evidently can move as fast as oceanic plates, but the latter probably spend a higher proportion of their time actually moving rapidly than do the former. For although the motional resistances of the two types of plate may be comparable, the subduction cycles of the two may be quite different. Put very briefly, because continental and oceanic lithospheres behave rather differently when approaching and meeting trenches, subduction of an oceanic plate will continue for much longer than subduction of an otherwise comparable continental plate. The result, according to Gordon *et al.*, is that "Statistically . . . plate motions sampled at a given instant of time, such as the present, are likely to show a negative correlation between plate velocity and continental area." □

100 years ago

There is much more than the name of Cambridge to remind one of its namesake at home. Its quiet air of studious retirement, its quaint buildings and tree-shaded walks have much of the mother-country about them. One or two features of the place, however, are characteristically American. Thus in the great library at Gore Hall, most of the work of receiving and distributing books is done by young women, and done, too, with a noiseless decorum and celerity worthy of all praise. A magnificent Memorial Hall to those graduates of Harvard who fell in the late Civil War bears witness in its crowded lists of names that culture and courage may go hand in hand.

While Harvard is necessarily the great centre of scientific research, much admirable work is done in Boston in the way of practically expounding science. The Institute of Technology has for its primary object the education of the community in these branches of scientific knowledge conducive to progress in the arts and industries of life. In pursuance of this aim the methods of tuition are so practical and thorough that the results must be felt far beyond the industrial circles. Established mainly through the enlightened zeal of the present venerable President of the National Academy, Prof. W. B. Rogers, it began a few years ago to languish, but its founder has recently come back to its rescue, throwing himself into its affairs with all his old heartiness and kindness until, freshened and stimulated by his influence, it is once more shooting up into lusty vigour.

From *Nature* **21**, 18 Dec., 150; 1879.

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articles

Deuterium concentration in the early Solar System: hydrogen and oxygen isotope study

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D/H ratios in the unequilibrated chondrite Chainpur range up to 9×10^{-4} . Corresponding ^{17}O contents are 1% higher than in other LL chondrites, with varying ^{18}O contents. These results are discussed in terms of low temperature condensation of water and early irradiation by intermediate energy protons. The latter explanation is favoured.

THE determination of universal deuterium abundances is a major limiting factor on the density of the Universe and helps to determine a model for its expansion¹⁻³. Several estimates of the D/H ratio in the protosolar nebula have been made on the basis of experimental data^{4,5} and theoretical considerations^{6,7}. Recent results give the average ratio as $1.9 \pm 1.1 \times 10^{-5}$ (ref. 8). Present theories on stellar evolution suggest that the thermonuclear reactions taking place at the centre of the Sun totally convert the deuterium into ^3He . Calculations show that the D/H ratio should be $\sim 10^{-17}$; direct measurements give a value of $< 2.5 \times 10^{-7}$ at the solar surface⁹ and $< 3 \times 10^{-6}$ in solar wind¹⁰. Hence only planets and meteorites could have preserved some trace of deuterium cosmic abundance. Evolution of the primitive atmospheres on terrestrial planets has undoubtedly altered the initial abundance of deuterium during outgassing of the planet and preferential losses of light hydrogen in the upper atmosphere. On the Moon, present nuclear phenomena (implantation of solar wind protons, *in situ* production of spallation deuterium by galactic cosmic rays) are strong enough to blank out any trace of the primordial isotopic ratio.

In 1954, Boato¹¹ measured the D/H ratios in some type I and II carbonaceous chondrites. His work did not completely determine the amount of terrestrial pollution in these very fragile materials, which contain a large amount of partly exchangeable water. However, he observed a 40% variation of the D/H ratio and showed that some fractions of Orgueil and Mokoia contained hydrogen enriched up to 20% relative to SMOW, the upper range of deuterium enrichment (155.76 p.p.m.) for most terrestrial hydrogen components.

Hence these fractions were probably traces of genuine extra-terrestrial D/H ratios. They show an approximately 10-fold enrichment relative to the primordial D/H estimate defined above. Little work was then done until, in 1975, we studied ordinary chondrites to ascertain whether this enrichment was an overall feature of telluric objects^{12,13}. Our analytical procedure was much more sensitive than Boato's, allowing isotopic measurements on 0.1–20 mg samples. This makes it possible to test isotopic homogeneity on these samples, which are generally < 1 g.

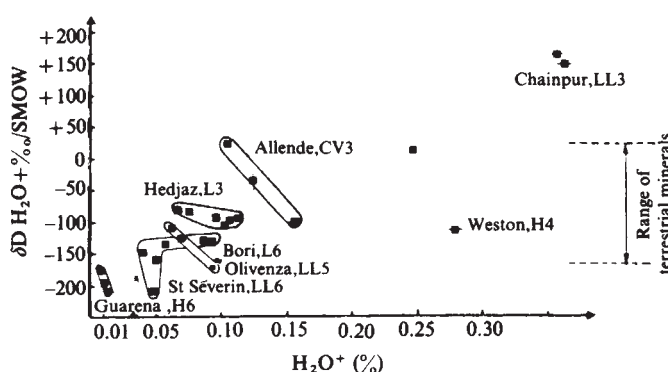


Fig. 1 D/H and hydrogen contents reported as H_2O^+ for various chondrites showing an overall positive correlation.

The results are summarised in Fig. 1, where D/H is plotted against H_2O^+ content (water extracted at $T > 200^\circ\text{C}$). They show a D/H variation from 124 p.p.m. for Guarena (LL6) to 220 p.p.m. for Chainpur whose conventional petrographic type is LL3 but whose matrix carbon content and chondrules' characteristics are very similar to those of carbonaceous chondrites. All these values are strongly enriched relative to the

cosmic abundance (a factor between 6 and 11) and comparable to terrestrial values. On the other hand, we observe large variations between meteorites, and inside a single meteorite. These variations are particularly striking in the case of Chainpur. These features have been discussed¹²⁻¹⁴ in the framework of accretion models of parent bodies and subsequent metamorphism of equilibrated chondrites. On the whole the results agree with the hypothesis of water adsorption on previously condensed grains, at temperatures varying from 180 to 230 K and total pressures of 10^{-2} – 10^{-4} atmospheres, increasing with decreasing temperature, and of a corresponding increase of the D/H ratio due to a rapid increase of the fractionation coefficient $\alpha_{\text{D H}_2\text{O}-\text{H}_2}$ with decreasing temperature. Unequilibrated chondrites would completely retain these low temperature features whereas metamorphosed chondrites could have been depleted in water and deuterium by outgassing of variable quantities of deuterium enriched water (Fig. 2).

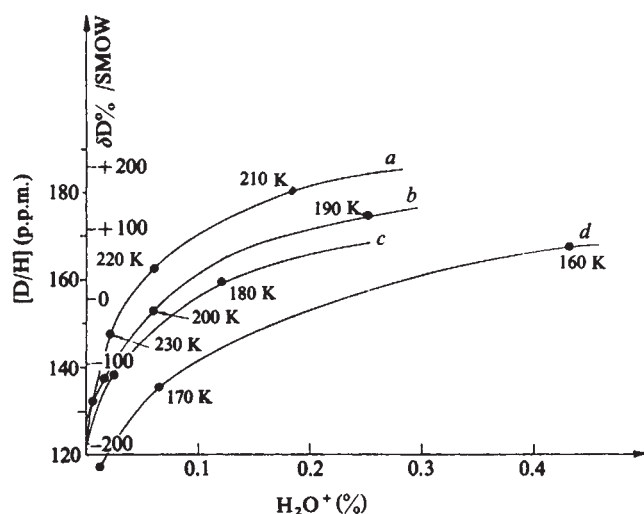


Fig. 2 Model of water adsorption on grains with fractionation $\text{H}_2\text{--H}_2\text{O}_{\text{sol}}$ at various total pressures using data of Sill and Wilkening for total water adsorption and Richet *et al.* for $\alpha_{\text{D H}_2\text{--H}_2\text{O}_{\text{sol}}}$. Curves: a $P_T = 10^{-2}$ atm, $(\text{D}/\text{H})_{\text{H}_2} = 25$ p.p.m.; b, $P_T = 10^{-3}$ atm, $(\text{D}/\text{H})_{\text{H}_2} = 19$ p.p.m.; c, $P_T = 10^{-4}$ atm, $(\text{D}/\text{H})_{\text{H}_2} = 15$ p.p.m.; d, $P_T = 10^{-5}$ atm, $(\text{D}/\text{H})_{\text{H}_2} = 11$ p.p.m.

This model, while satisfactory for the whole meteorite sample, implied drastic temperature and pressure variations to explain the total range (140–218 p.p.m.) observed in a 1-g sample of Chainpur. We therefore decided to measure ^{18}O and ^{17}O abundances on the same samples, to look for possible relationships between deuterium contents and the location of these samples in a $\delta^{17}\text{O}$ – $\delta^{18}\text{O}$ diagram¹⁵. (δD , $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ are defined as: $\delta = (R_{\text{sample}}/R_{\text{SMOW}} - 1) \times 1,000$, where $R = \text{D}/\text{H}$, $^{17}\text{O}/^{16}\text{O}$, $^{18}\text{O}/^{16}\text{O}$.)

Analytical procedure

Analyses have been made on five samples (chondrules and matrix) separated by gentle crushing. The chondrules were then picked up and washed from matrix debris with acetone in an ultrasonic tank. The Chainpur matrix originally contained debris of chondrules¹⁶ so that the 'matrix' sample is only the most enriched in matrix material in a petrological sense. Hydrogen was extracted by heating with an electrical resistance furnace.

Our previous results¹²⁻¹⁴ were obtained by a vacuum extraction technique. For the present study, the extracted gases were carried by an helium flow through a vanadium furnace heated at 800 °C where the reduction of the hydrogen compounds takes place, and then introduced directly into the ion source of the mass spectrometer after selective diffusion of hydrogen through

palladium heated at 450 °C. The gas mixture flows along a tube made of silver–palladium alloy (50 mm long and 0.08 mm thick) which is directly sealed to the line of the mass spectrometer. The flow of helium ($\sim 0.25 \text{ cm}^3 \text{ s}^{-1}$) is kept small relative to the diffusion rate of hydrogen through the silver–palladium tube ($\sim 1 \text{ cm}^3 \text{ s}^{-1}$). Thus the variation of the isotopic composition of hydrogen with rising temperature is continuously monitored.

Systematic calibrations are made to measure the absolute sensitivity of the whole apparatus to hydrogen and D/H ratios, using known amounts of hydrogen with a known D/H ratio. The method has been checked by the analysis of a terrestrial phenigite provided by O'Neil with $\delta\text{D} = -39\%$. Three measurements of this sample gave $-40 \pm 2\%$ with a flat stepwise heating D/H release pattern between 200 and 1,200 °C.

After hydrogen extraction, oxygen was recovered by the BrF_5 technique, purified through a 5 Å molecular sieve, collected and analysed as O_2 in Micromass 602C double collecting mass spectrometer. We checked that no impurities were present, which could contribute to masses 33 or 34 (essentially NF_3 which would appear also at mass 52 (ref. 17)). Traces of CF_4 were occasionally present, which could not modify the 33 or 34 currents.

Table 1 shows the results as conventional H_2O^- (hydrogen extracted below 200 °C) and H_2O^+ (>200 °C) contents. For samples A and B, H_2O^+ has been extracted by heating between 200 and 1,150 °C at a rate of about $15 \text{ }^\circ\text{C min}^{-1}$. For samples C, D and E temperature steps have been observed. Table 1 and Fig. 3 show that deuterium rich fraction 3 is extracted between 450 and 750 °C. The relatively high values of the D/H ratio observed in the hydrogen extracted at $T > 750 \text{ }^\circ\text{C}$ may, at least partly, reflect a tailing effect of the whole extraction line, mainly in the vanadium furnace and the palladium membrane. In contrast, it is worth noting that: (1) that fraction 2 ($200 < T < 450 \text{ }^\circ\text{C}$) is not enriched in deuterium compared with fraction 1

Table 1 Oxygen and hydrogen isotope composition of Chainpur samples

Samples	H_2O (10^{-4} g per g)	D/H (p.p.m.)	$\delta^{17}\text{O}\%$ (SMOW)	$\delta^{18}\text{O}\%$ (SMOW)
A $m = 10.7$ mg				
1 chondrule				
(a) H_2O^- 20–200 °C	5.3	165		
(b) H_2O^+ 200–1200 °C	5.5	326	+3.2	+4.9
B $m = 15.2$ mg				
6 chondrules				
(a) H_2O^- 20–200 °C	9.2	153		
(b) H_2O^+ 200–1200 °C	6.0	201		
C $m = 21.1$ mg				
10 chondrules				
(a) H_2O^- 20–200 °C	4.7	157		
(b) H_2O^+ 200–450 °C	3.0	155		
450–750 °C	0.5	846		
750–1200 °C	0.7	242		
H_2O^+ mean	4.2	252	+5.6	+7.5
D $m = 9.1$ mg				
8 chondrules				
(a) H_2O^- 20–200 °C	3.9	161		
(b) H_2O^+ 200–450 °C	2.7	154		
450–750 °C	1.3	471		
750–1200 °C	0.1	199		
H_2O^+ mean	4.1	256	+4.2	+5.4
E matrix				
(a) H_2O^- 20–200 °C	24	156		
(b) H_2O^+ 200–450 °C	11	146		
450–750 °C	3.4	672		
750–1200 °C	1.8	350		
H_2O^+ mean	16.2	279	+3.5	+4.1

Oxygen data refer to whole sample after hydrogen extraction by step heating experiments.

(H_2O^-); and (2) that no correlation is observed between the isotopic ratios of fractions 2 and 3 (Fig. 3).

The values of D/H measured in fraction 3 of samples *D* and *E* are the highest ever recorded in natural samples. D/H ratios up to 300 p.p.m. have been measured¹⁸ in a lunar rock irradiated by cosmic rays, where the high values of D/H were observed in fractions extracted at $T < 470^\circ\text{C}$ in a background of normal, deuterium free, lunar hydrogen of solar-wind origin.

The $\delta^{17}\text{O}/\delta^{18}\text{O}$ ratio (Table 1 and Fig. 4) diagram¹⁵ enables a distinction to be made between physicochemical (mass dependent) fractionations, which correspond to variations along a straight line of gradient $\frac{1}{2}$ and nuclear effects which define other correlations or possibly no correlation at all.

Chainpur results are variable, and, except for sample *A* plot well above the mean value for LL chondrites measured by Clayton *et al.*¹⁷ defining approximately a straight line of gradient $\frac{1}{2}$ translated by +0.5% in $\delta^{17}\text{O}$ relative to the LL straight line. Hence Chainpur samples contain more ^{17}O than any other object in the Solar System or less pure- ^{16}O component. This is related to the fact that Chainpur is the only LL3 analysed to date and work in progress suggests that even larger oxygen isotope heterogeneities exist in this meteorite and other type 3 chondrites.

Discussion

There are two main explanations for such results, one relating to physicochemical fractionations and one to nuclear effects and they will probably have to be mixed to some extent.

The driving mechanisms are: temperature variations for the physicochemical fractionation, mixing with presolar grains or heavy energetic irradiations for nuclear effects (oxygen, magnesium, neon, and so on).

Until the discovery of the $^{17}\text{O}/^{18}\text{O}$ anomalies in the meteorite Allende¹⁶ oxygen isotope variations in meteorites had been mainly explained in terms of physicochemical fractionations. The few D/H results, since deuterium formation is attributed almost exclusively to the big bang^{1,2}, have also been explained by physicochemical fractionations¹⁹ eventually associated with mixing to deuterium free solar wind¹⁰. The departure from physicochemical effects can be demonstrated if more than two isotopes are present (oxygen) and is much more difficult for elements such as hydrogen which have only two stable isotopes. The main indication for an isotope anomaly in these elements would be the size of the isotopic effect and an eventual correlation with other definitely nuclear effects.

Although irradiation models have recently been considered for oxygen²⁰⁻²², the variations are so large and most of them so well correlated on a $\delta^{17}\text{O}/\delta^{18}\text{O}$ diagram^{15,17} that the hypothesis of presolar grains resulting from the explosion of a nearby supernova¹⁵ and carrying pure ^{16}O , is still more convincing. Chainpur would be the least contaminated by this pure ^{16}O component. Isotopic variations among samples *C*, *D* and *E* could be attributed mainly to physicochemical fractionations whereas sample *A* would reflect a different proportion of ^{16}O component. Alternatively this departure from the straight line with gradient $1/2$ could be due to ^{17}O and ^{18}O created from spallation reactions.

Temperature dependent fractionation

The step heating process reveals for hydrogen, inside a given fraction, large heterogeneities with enormous D/H ratios. To account for these ratios in the framework of water adsorption at low temperatures, we have to consider temperatures down to 110 K. In a simple black-body model this corresponds to a distance of about 3 AU, the location of the asteroid belt, but this cannot be used as a definitive argument since Wetherill²³ demonstrated that the asteroid belt is a kind of gravitational well for small objects coming from a large region of the Solar System. Measurement on interstellar clouds from the Orion nebula²⁵

shows an enormous D/H ratio in the HCN molecule, more or less correlated with the temperature down to 10 K, which could be obtained by equilibrium or kinetic, temperature dependent, fractionations.

Hence the observed D/H distribution in Chainpur may be the result of a very complex mixing of hydrogen compounds condensed at various temperatures in the solar nebula or even remnants of the interstellar medium. However, the fact that D/H fractionations in Orion depend on the temperature down to 10 K indirectly suggests that any change to a higher temperature would redistribute the D/H ratios. In particular in chondrules, which were strongly heated and probably fused, we should have uniform D/H ratios. This, together with the medium temperature range (450–750 °C) at which we obtain the D/H peak is against the survival of volatile molecules and in support of the creation of anomalies after the chondrules formation.

Early irradiation model

Deuterium can be created in various environments. Besides the big bang which is the most favoured model, two hypotheses directly relate to the early history of the Solar System. The first one is the effect of shock waves in supernovae envelopes²⁷. The conditions postulated for the production of deuterium in such envelopes are far different from those considered for the production of the isotopically anomalous presolar grains and require energies $> 10^{53}$ erg, which have not been observed. Also our deuterium enrichments have been found in the ^{17}O and ^{18}O

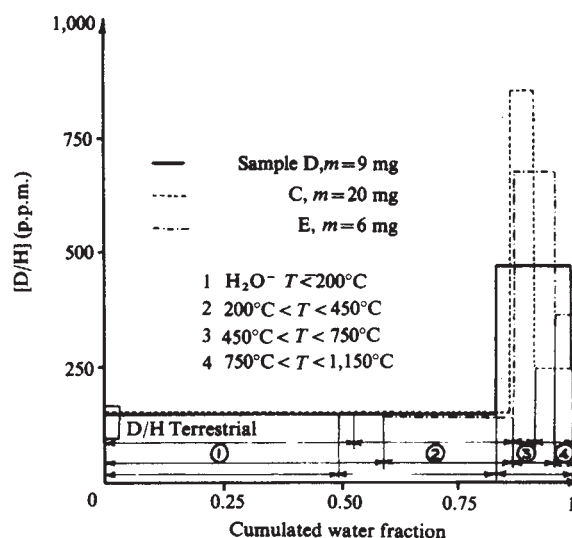


Fig. 3 D/H and water contents extracted during stepwise heating experiments of Chainpur samples, corresponding to the data of Table 1.

richest material which is exactly the opposite of what is expected of presolar grains originated from supernovae. The second involves an early irradiation within the Solar System. This hypothesis is rejected by Reeves *et al.*² and seriously questioned by Epstein *et al.*²⁶ for deuterium production, it also has been considered to explain neon, oxygen, magnesium and other isotope anomalies discovered in the 1970s. It is finally rejected by Clayton *et al.*²¹ and proposed with special and somewhat *ad hoc* limitations by Lee²². However, our very special deuterium anomaly leads us to reconsider it.

If this model proves to be reasonable, we shall also have to consider its logical extension: not only the D/H ratio is heterogeneous in Chainpur but every chemical and petrological feature and this is due to the fact that it is an unequilibrated

chondrite. If the early irradiation affected Chainpur, it possibly also affected the parent material of larger objects such as the Earth where the deuterium was largely redistributed and homogenised. Hence this irradiation can be responsible for the production of a large proportion of the deuterium on Earth and other large objects even if the original signature has been lost by homogenisation. If energetic and cosmochemical evidence is not violated this hypothesis cannot be ruled out.

In such a model first we have to estimate which part of the deuterium measured on our experiments is the spallation product: that corresponding to the peak of very high D/H, the deuterium excess relative to the equilibrium content relative to the nebula at a given temperature, or the whole deuterium content. Given the importance of the high D/H peak, the three possible choices give results comparable within an order of magnitude. For simplicity, we have reported in Table 2 the deuterium quantities corresponding to H_2O^+ (hydrogen extracted at $T > 200^\circ\text{C}$).

We also have to decide what is the possible contribution of terrestrial contamination: for all our experiments on chondrites^{12,13}, it has been considered negligible above 200°C .

Several spallation reactions by protons produce deuterium with reasonable yields. For a power law energy spectrum ($d\phi/dE = K e^{-\gamma}$ with $2 < \gamma < 5$) and low energy cutoffs of < 10 MeV, these reactions typically have effective cross-sections of the order of 0.1 b (ref. 21). The most efficient should be on oxygen and iron targets in the solid state and helium in the gaseous state. We cannot get such high D/H in the gas without unreasonably high fluences. In the solid, due to the high (Fe + O)/H ratio, necessary fluences are much lower. They are listed in Table 2 where they range from 10 to 10^{20} cm^{-2} . These fluences could be notably reduced (down to a factor of 10) if capture of secondary neutrons produced by spallation were also considered³. However, for this process to be really efficient, we need thermalised neutron fluences up to 10^{20} cm^{-2} which should show up on the isotopic composition of elements such as gadolinium, which should be looked for in Chainpur. Until then we shall assume that the necessary proton fluence is 10^{19} – 10^{20} cm^{-2} which is comparable to that considered by Clayton *et al.*²¹ to produce the ^{26}Mg anomaly in Allende but much less than the fluence needed to produce the maximum oxygen anomaly (10^{23} – 10^{25} cm^{-2} according to the scenario^{21,22}).

Energy limitations

The next step is to decide what kind of system and what global mass was irradiated by such an effect. If the spallation deuterium is restricted to the peak and not found in other objects, this means that the irradiation is restricted to the surface of tiny objects like Chainpur parent body and the energy problem will not be too serious. The isotopic and chemical difficulties are reviewed later. However, this is an *ad hoc* scenario and this proton fluence is more likely to come from the main source of energy of the system which is the young Sun. Then all the matter exposed between the Sun and the location of Chainpur conden-

sation (and possibly further) has to be considered. The period which we must consider is that during which solids condensate, planetary bodies accrete and residual nebula is swept away, and it might be very short (10^7 – 10^8 yr). The period of T Tauri radiation itself might be much shorter (10^6 yr)²⁹. Possible scenarios, such as nuclear and energy needs, strongly depend on when precisely the T Tauri phase occurs in the longer period if a planet is already formed then the effects of the radiation will be only surficial and vanish eventually by mixing. This is particularly important for the jovian planets. If the nebula has already been swept away, then the stopping distance is much larger, the mass involved and the energy needed considerably reduced. The careful examination of deuterium contents may then set the average accretion time of planets relative to the T Tauri phase and to each other.

Practically, if we want to apply a 10^{20} cm^{-2} fluence to the jovian planets or to a nebula of 10^{-5} – 10^{-3} atmospheres total

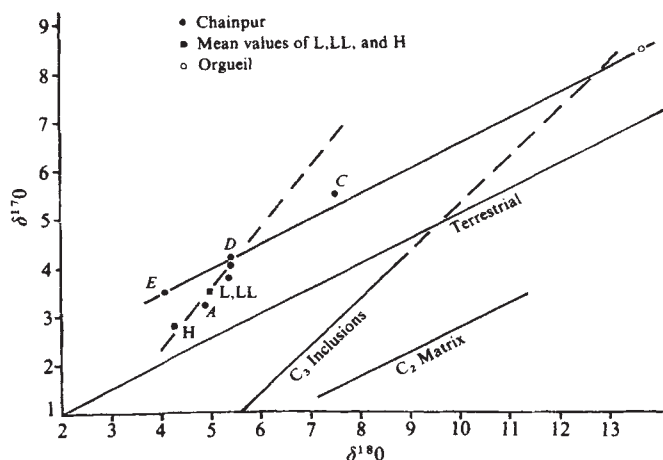


Fig. 4 $\delta^{17}\text{O}/\delta^{18}\text{O}$ plot of the isotopic composition of Chainpur samples, corresponding to the data of Table 1. ■, data from ref. 21.

pressure extending over ~ 1 AU, we have to consider a total mass of 10^{-3} – $2 \times 10^{-1} M_\odot$, which leads to totally unreasonable energy dissipation.

In contrast, the hypothesis which suggests that the deuterium content of the terrestrial planets could largely be due to spallation is reasonable from an energetical point of view. The global D/H ratios in meteorites and the Earth vary from 140 to 220 p.p.m. and are thus very similar. The Earth probably contains most of the total deuterium estimated at 1.5×10^{43} – 1×10^{44} atoms. This number cannot be more than doubled if we take into account all the terrestrial planets. If this whole mass was irradiated in its dust form to produce most of its deuterium, we can calculate the total energy needed, mostly lost by ionisation. The total mass corresponds roughly to 5×10^{50} atoms. Taking a mean ionisation cross-section of $10^{-26} \text{ erg cm}^{-2}$ as an upper limit and making calculations similar to Lee's²², we find a total energy of 5×10^{44} erg (of which 5×10^{39} erg only are dissipated by the formation of deuterium). This is roughly 10^{-4} of the total gravitational energy of the Sun. The only remaining problem is the question of the stopping distance; which strongly depends on the particle energy. For example, the residual trajectory is 5 – $15 \times 10^{-2} \text{ g cm}^{-2}$ for a 10 MeV proton and 55 – 130 g cm^{-2} for a 500 MeV proton in hydrogen and oxygen respectively^{30,31}. For a 500 MeV proton, the energy dissipated by nuclear reactions would increase to 2.5×10^{44} erg but the total energy loss would eventually decrease because the stopping power is less. The total flux of such protons ($\phi \times t = 10^{20} \text{ cm}^{-2}$) across a sphere of 1.5 AU radius would amount to 2×10^{44} erg, roughly one-third of the total energy need. Whether this intermediate range of energy, postulated by

Table 2 Total deuterium contents (atoms per g), corresponding to H_2O^+ (extracted above 200°C) and corresponding fluences needed for protons of $E > 10$ MV

Sample	D in H_2O^+ (atoms per g)	$I \times t$ (protons $\text{cm}^{-2} \text{ s}^{-1}$)
A	12×10^{15}	3.3×10^{19}
B	8.1×10^{15}	2.2×10^{19}
C	7.1×10^{15}	2.0×10^{19}
D	7.0×10^{15}	1.9×10^{19}
E	30.2×10^{15}	8.4×10^{19}

Fowler *et al.*² is realistic is debatable but it is energetically reasonable. It provides much longer travel distances, which would allow the protons to penetrate deeply (~ 40 cm) into planetesimals, and not only dust, and also to travel 1 AU into a $3 \times 10^6 \text{ g cm}^{-3}$ (3×10^{-8} atm) nebula.

Cosmochemical constraints

The total amount of deuterium formed is only 3×10^{-8} of the estimated protosolar deuterium. Hence the global deuterium cosmology is not strongly modified inasmuch as only 7–30% of the matter is supposedly reprocessed in stellar systems. But the early irradiation can have very strong effects on other elements in the irradiated materials, especially on Li, Be, B, rare gases, magnesium (^{26}Mg) and also on rarer nuclei such as ^{50}V , ^{92}Nb , ^{138}La , ^{180}Ta (refs 21, 22). As most of the latter nuclei still have to be investigated, we only consider the problem of light elements and mention the rare gases limitations.

We stress that our deuterium anomaly does not apply to the same type of material as Allende's inclusions (as is reflected by the mineralogy and also by the ^{17}O – ^{18}O results), but the postulated fluences are similar to that proposed by Clayton *et al.*²¹. They would produce rather significant amounts of Li, Be and B by spallation on oxygen, carbon and nitrogen³² and also large amounts of rare gases among which the already discussed ^{22}Ne , and ^{36}Ar and ^{38}Ar from ^{39}K and ^{41}K respectively²¹. They could also produce ^{17}O and ^{18}O by spallation on the Si group, with rather small cross-sections (~ 10 mb), but, because of the large abundance of the Si group about 10 times more efficient than the destruction reactions.

These spallations (mainly on Si and Mg), could produce 10^{15} – 10^{16} atoms g^{-1} of ^{17}O and ^{18}O which corresponds to variations in $\delta^{17}\text{O}$ of 0.15 to 1.5‰ and in $\delta^{18}\text{O}$ of 0.03 to 0.3‰. The ^{17}O variations approximately match the total offset relative to a straight line with a gradient of $\frac{1}{2}$ whereas the ^{18}O variations are much smaller than the observed values. This can reflect the result of two different processes: (1) the proton irradiation, acting mainly on ^{17}O and (2) the effect of mass dependent fractionations, occurring, for example, during the formation of the chondrules, which would bring the isotopic composition of the condensed phases from $\delta^{18}\text{O} \sim 4\%$ closer to the nebular composition (+13 to +14‰ after the intercept between Chainpur line and Allende's inclusions line).

Consider then the Li, Be and B: using cross-sections of 10–20 mb (ref. 29), we calculate for our maximum influence of 10^{20} cm^{-2} a production of about 0.3 p.p.m. of Li and about twice as much B. The production of Be should approach 0.01 p.p.m. If we compare this to the observed concentrations^{33–35} which range from 1 to 3 p.p.m. for Li and B, we have to account for a 10–60% increase, which from an elemental point of view is perfectly acceptable.

The most severe limitations come from the isotopic composition as there are large differences between meteoritic and spallation-produced $^7\text{Li}/^6\text{Li}$. Observed meteoritic $^7\text{Li}/^6\text{Li}$ are up to now equal within a few per cent to 12.5 including Chainpur³³ whereas spallation Li has $^7\text{Li}/^6\text{Li}$ ranging from ~ 3 to 7 depending on the energy range. The data of Chainpur show that we should expect a decrease of $^7\text{Li}/^6\text{Li}$ from 12.7 to 12 or 11.4, which should be perfectly measurable.

Now our model attempts to explain essentially all the observed D/H ratios in terrestrial objects and for them we need comparable fluences. For the Sun and stars we have only a lower limit for $^7\text{Li}/^6\text{Li}$ and the only accurate numbers are for terrestrial objects, so the observed homogeneity may be the result of mixing in almost constant proportions of lithium from three sources: ^7Li from the big bang, ^7Li and ^6Li from galactic cosmic radiation, producing only a few per cent variation. Note that this remarkable homogeneity remains to be satisfactorily explained even if we remove the third component.

The postulated fluences produce too much ^{22}Ne in the solid as discussed by Clayton *et al.* For Chainpur (0.1% K) the ^{36}Ar production should be $0.5\text{--}5 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$, which satisfactorily matches the results obtained by Podosek³⁶ (0.85 to $>1.18 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$). The $^{36}\text{Ar}/^{38}\text{Ar}$ spallation ratio should vary between 15 and 70 according to the energy spectrum²¹. The ratio observed by Podosek is 3.5–4.0 in an irradiated sample. If ^{38}Ar is corrected for ^{38}Ar produced from ^{37}Cl (^{37}Cl (n, γ , β) ^{38}Ar) as for Karoonda^{36,37}, the ratio is 10–14, very different from the solar value. However, other results on Chainpur³⁸ do not confirm this result. Also, the mechanisms by which the Earth can rebuild a solar $^{36}\text{Ar}/^{38}\text{Ar}$ after the early irradiation remain to be imagined precisely.

Conclusion

In the meteorite Chainpur we have found very large anomalies in deuterium contents associated with small enrichments in ^{17}O and larger variations along a straight line with gradient 1/2 in a $\delta^{17}\text{O}/\delta^{18}\text{O}$ diagram. Deuterium results can be explained by a temperature dependent model of fractionation in a wide range of low temperatures, but an early irradiation during the T Tauri phase is presently favoured, which could explain most of the hydrogen and oxygen isotope features. The extension to C_1 chondrites is very difficult because the deuterium amounts are 20 times larger.

We thank P. Pellas for samples. J. Audouze for his kindness, and J.-F. Minster, C.-J. Allegre and E. Roth for helpful comments.

Note added in proof: Since this report was written Clayton and Mayeda found very similar results to the Chainpur samples on a LL3 chondrite recovered from Antarctica (ANS) (see ref. 40).

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Nebular condensation of Ga, Ge and Sb and the chemical classification of iron meteorites

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Key parameters for classifying iron meteorites into genetic groups are concentrations of Ga and Ge. Their taxonomic value results from the combination of very wide concentration ranges in iron meteorites as a whole, with very narrow concentration ranges in most individual groups. The very wide intergroup ranges result from solar nebular condensation in widely varying conditions. Recent calculations show that the nebular condensation temperature of Ge is lower than that of any other siderophile, and that Sb and Ga are the next most volatile siderophiles. The narrow intragroup ranges of Ga and Ge reflect minimal fractionation during the crystallisation of cores, larger intragroup ranges for Sb result from a solid/liquid distribution ratio near 0.4.

Only during the past 20 yr have meteorite researchers developed a genetic classification for iron meteorites¹⁻⁵. This system, often called the chemical classification, is based on a combination of metallic structure and the concentration in the metal of several siderophile (metal-preferring) elements, most especially Ga and Ge⁶⁻⁸. The Ga-Ni and Ge-Ni fields occupied by the 13 groups of iron meteorites are shown in Fig. 1. Although the total concentration ranges among all meteorites are 1,600 and 400,000 for Ga and Ge, respectively, only the two groups IAB and IIICD have maximum/minimum ranges >2. There is evidence which indicates that the groups having wider ranges were never fully molten; their large Ga and Ge ranges may have been established during nebular fractionation^{9,10} or partial melting processes¹¹. Most evidence favours the view that the remaining 11 groups formed in planetary cores that were once fully molten^{12,13}.

The explanation for the Ga-Ge quantisation has been sought for the past two decades. Anders^{14,15} and Scott¹² attributed the large concentration ranges to nebular volatility, but were not able to explain why other siderophilic elements that appeared to be equally or more volatile did not exhibit similar distributions. Wai *et al.*¹⁶ examined the possibility that the low Ge contents could have resulted from preferred partitioning into silicate (mantle) materials; their experiments showed that Ge was highly siderophile in temperature and redox conditions appropriate to core formation.

Non-nebular fractionations have produced large intragroup concentration range of some elements (such as, Ir, Os and Re ranges of a factor 5,000 are observed in group IAB), requiring extreme care when estimating group means. To calculate intergroup ranges we have reviewed published data^{17,18} and used treatments involving fractional crystallisation equations or weighting to achieve precise mean compositions¹⁹, then calculated mean abundances relative to Ni. The six highest

maximum/minimum abundance ranges are 15,400, 890, 138, 55, 36 and 11.4 for the elements Ge, Ga, Sb, As, Cu, and Au, respectively. We have previously calculated 50% condensation temperatures for several moderately volatile elements and showed that there was an approximately monotonic decrease in the ordinary-chondrite/CI-chondrite abundance ratio with decreasing nebular condensation temperature²⁰. As expected, the maximum/minimum abundance ranges in iron meteorites are also negatively correlated with our previously published condensation temperatures, but an important exception is that the Ga range is higher than that of Sb despite the fact that our previously calculated Ga condensation temperature was 150 K higher than that for Sb.

Nebular equilibria

The correctness of equilibrium condensation calculations depends largely on the quality and completeness of available thermodynamic data of the relevant gaseous and condensed forms of the element under consideration. For example, if lack of data results in exclusion of a stable gaseous form of an element from the calculation, the calculated condensation temperature will be too high. If a minor or trace element condenses in solid solution with a major mineral phase, calculation of a correct condensation temperature requires inclusion of an appropriate activity coefficient (γ) of the element in the host phase. In most previously reported condensation temperatures γ was taken equal to unity, that is, ideal solution behaviour was assumed. In our published calculations²⁰ we estimated γ values that were assumed to be independent of temperature.

In fact, the activity coefficient depends both on temperature and composition. The relationship of γ to the temperature T , the partial molar heat of solution $\Delta\bar{H}_s$ and the excess partial molar entropy of mixing $\Delta\bar{S}^{xs}$ is given in standard thermodynamics texts²¹:

$$\ln \gamma = \frac{\Delta\bar{H}_s}{RT} - \frac{\Delta\bar{S}^{xs}}{R} \quad (1)$$

where R is the gas constant. Based on available data, we have estimated the activity coefficients of the six most volatile siderophilic trace elements in Fe-Ni according to equation (1). The partial molar quantities for each trace element given in Table 1 are for a dilute solid solution with the mole fraction (X) of the element approaching zero; the Fe:Ni mole ratio is assumed to be 12. The quality and suitability of the available thermodynamic data vary, as described below.

The activity coefficient of Au in Ni ($\gamma_{\text{Au[Ni]}}$) at 1,150 K approaches 10 as X_{Au} approaches zero²². At 1,123 K $\gamma_{\text{Au[Fe]}}$ data are available only for X_{Au} from 1.0 to 0.45; the trend follows closely that of $\gamma_{\text{Au[Ni]}}$. Therefore, the $\Delta\bar{H}_s$ and $\Delta\bar{S}^{xs}$ for Au in Fe-Ni were assumed to be identical with those for the Au-Ni system. Activity coefficients of Cu in solid Ni or Fe are not available, but $\gamma_{\text{Cu[Ni]}}$ and $\gamma_{\text{Cu[Fe]}}$ at $X_{\text{Cu}} = 0$ have been reported for liquid systems at 1,823 K (ref. 22). Solid phase γ

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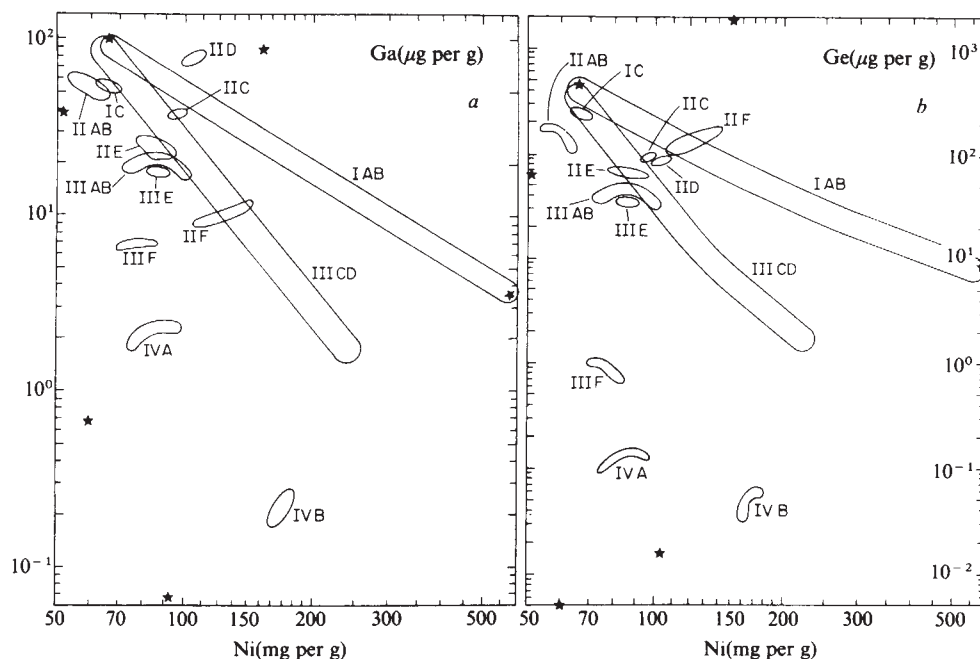


Fig. 1 log-log Ga-Ni (a) and Ge-Ni (b) diagrams; note differences in the logarithmic scales. The large concentration ranges in the non-magmatic groups IAB and IIIICD reflect the fact that they were not subjected to the homogenisation associated with core formation. The 11 groups that formed on cores show quantised Ga and Ge ranges (factors ≤ 1.7) and minimal overlap. Stars show the locations of the iron having maximum and minimum concentrations of these three elements; two are members of group IAB and the remainder ungrouped.

values were calculated based on iron meteoritic evidence that the equilibrium partition coefficient of Cu between liquid and solid Fe-Ni is close to unity, that is, the γ value of Cu in solid Fe-Ni should be close to that for liquid Fe-Ni (ref. 19). From these data we estimated the Cu[Fe-Ni] partial molar quantities.

We estimated partial molar quantities for Ge in Fe-Ni from infinite dilution $\gamma_{\text{Ge}}[\text{Fe}]$ and $\gamma_{\text{Ge}}[\text{Ni}]$ values at 1,800 K (refs 23, 24), and from a solid/liquid distribution coefficient of 0.89 observed in low-Ni group IIIAB iron meteorites¹⁹. Extrapolation below $X_{\text{As}} = 0.11$ yielded $\gamma_{\text{As}}[\text{Fe}] = 0.5$ at infinite dilution at 1,573 K (ref. 25). We estimated the partial molar quantities for As in solid Fe-Ni from these data and from the As solid/liquid distribution coefficient of ~ 0.41 inferred for group IIIAB (ref. 19). Partial molar quantities for these four siderophiles are based on high-temperature experimental data from dilute binary solutions and from meteoritic measurements. A major source of uncertainty in this application is the assumption that $\Delta\bar{H}_s$ is temperature independent over a wide temperature range. An error of 1–2 kcal mol⁻¹ in the estimated $\Delta\bar{H}$ values would change the 50% condensation temperatures by 10–30°. Our estimated uncertainties are of this order.

The enthalpies of formation in solid Ga-Fe and Ga-Ni systems have been reported to exhibit large negative deviations with maximum values of -8.5 kcal mol⁻¹ (ref. 26) and -11.5 kcal mol⁻¹ (ref. 27), respectively. Hultgren *et al.*²² give a $\gamma_{\text{Ga}}[\text{Ni}]$ at 1,223 K of 3.3×10^{-3} at $X_{\text{Ga}} = 0.37$. Kelly and Larimer¹¹ suggested a lower $\gamma_{\text{Ga}}[\text{Fe-Ni}]$ of 3×10^{-3} which we find more in keeping with the lower $\Delta\bar{H}_s$ data for Ga-Fe, and our partial molar quantities were chosen accordingly.

Like its congener As, Sb forms stable compounds with Fe and Ni (ref. 21). The integral heat of solution and excess entropy of mixing of Sb in solid Fe at 800 K and $X_{\text{Sb}} = 0.45$ are

-2.3 kcal mol⁻¹ and -2 cal deg⁻¹ mol⁻¹, respectively²² and γ is 0.23 at $X_{\text{Sb}} = 0.3$ at 893 K (ref. 28). However, data for dilute Sb-Fe or Sb-Ni solid solutions are not available. Takahashi *et al.*²⁹ used $\gamma = 1 \times 10^{-3}$. This γ value at a typical Sb condensation temperature of ~ 900 K corresponds to a $\Delta\bar{H}_s$ of -14 kcal mol⁻¹ if $\Delta\bar{S}^{\text{xs}}$ is -2 cal deg⁻¹ mol⁻¹. This $\Delta\bar{H}_s$ is implausibly more negative than that estimated for As in Fe-Ni, thus we chose a smaller $\Delta\bar{H}_s$ value. The partial molar quantities of Sb given in Table 1 are more in keeping with the expectation that, given their closely similar electronic configurations, γ for Sb (radius ~ 1.6 Å) should be somewhat larger than that for As (radius ~ 1.5 Å) since As fits better into Fe-Ni lattice sites (mean radius ~ 1.28 Å)³⁰.

Because of the paucity of data, the relative uncertainties in the estimated partial molar quantities for Ga and Sb are $\sim \pm 30\%$. The errors in our calculated condensation temperatures for Ga and Sb caused by uncertainty in their activity coefficients are probably twice as large as those for Au, Cu, As and Ge. Despite the uncertainties in their activity coefficients we believe our calculated condensation temperatures for these elements are the most accurate allowed by currently available data.

We need to revise our previous results²⁰ regarding the distribution of Ga, Ge, and Sb among nebular gaseous species. Recently published data on GaOH(g)³¹ show it to be an important species, and Sears³² showed that GaCl(g) is also important. The nebular condensation of Cl is poorly understood, but it is more depleted than Ga in ordinary chondrites^{33,34}, suggesting that its condensation temperature is lower. Although the low abundance of Br limits its importance, GaBr(g) is also a stable nebular species³⁵. Based on the assumption that Cl condensed after Ga, we evaluate the relative abundances of Ga(g), GaOH(g), GaCl(g) and GaBr(g) from the

Table 1 Equilibrium nebular 50% condensation temperatures of some moderately volatile siderophilic elements

Element	Gaseous form	Condensed form (host)	$\Delta\bar{H}_s$	Solution data $\Delta\bar{S}^{\text{xs}}$	Condensation temperatures (K)	
					10^{-4} atm	10^{-6} atm
Au	Au(g)	Au (Fe-Ni)	6.0	1.5	1,225	1,074
As	As(g)	As (Fe-Ni)	-9.5	-2.5	1,157	1,012
Cu	Cu(g)	Cu (Fe-Ni)	11.0	2.0	1,037	910
Sb	SbS(g)	Sb (Fe-Ni)	-7.0	-2.0	912	767
Ga	GaCl(g)	Ga (Fe-Ni)	-9.0	-2.5	918	738
Ge	GeS(g)	Ge (Fe-Ni)	-16.0	-3.6	825	702

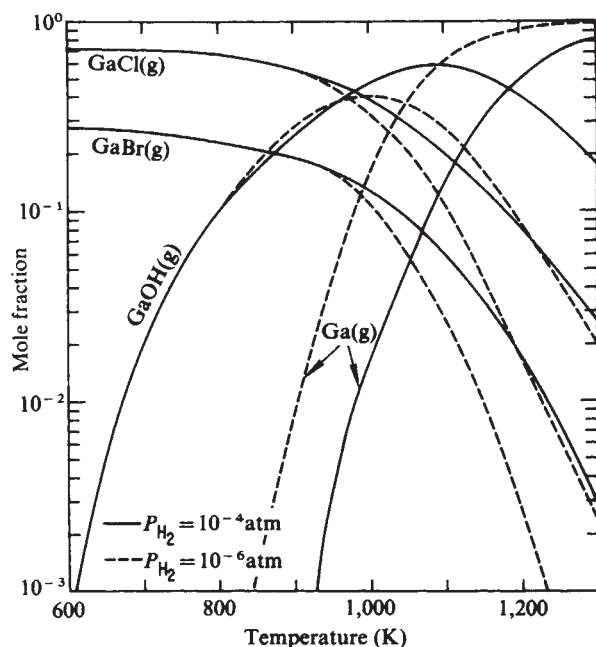
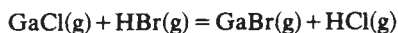
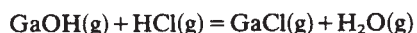
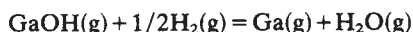


Fig. 2 Distribution of Ga among gaseous species in the solar nebula at pressures (P_{H_2}) of 10^{-4} and 10^{-6} atm. At high temperatures Ga is dominant, but with decreasing temperature first GaOH then GaCl become the most abundant gaseous forms.

following equilibrium processes:



The solar abundance of Cl (4,350, $\text{Si} = 10^6$) using in this calculation was taken from the Dreibus *et al.*³⁴ data or CI chondrites; the Br abundance (9.7, $\text{Si} = 10^6$) is based on unpublished data of B. Spettel, H. Wänke and G. W. Kallemeyn. The remaining abundances are from Cameron³⁶. The relative abundances of these four Ga species in the temperature range 600–1,200 K at nebular pressures (P_{H_2}) of 10^{-4} and 10^{-6} atm are shown in Fig. 2. The abundances of GaO(g) and Ga₂O(g) are insignificant in this temperature range. The condensation of Ga was calculated from:



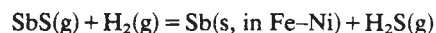
with the mole fraction of GaCl(g) corrected using the calculated distribution curve shown in Fig. 2.

The abundance of GeS(s) was underestimated in our previous study²⁰; we concur with Sears³² that it is the most abundant gaseous species of Ge. The condensation of Ge was calculated from available thermodynamic data^{37,38} by the following equilibrium process:



The partial pressures of GeS(g) used in our calculation were corrected for the minor amounts of GeSe(g) and GeTe(g) in the nebula³⁹.

Our²⁰ Sb condensation calculations are in need of revision to allow for the fact that SbS(g) is more abundant than Sb(g) at temperatures below 1,150 K. The condensation of Sb was recalculated from free-energy functions³⁹ and ΔH_f° (298 K)⁴⁰ data:



The condensation processes for the other three siderophiles Au, Cu, and As as discussed previously²⁰ seem to be correct, but condensation temperatures have been recalculated to include the temperature dependent γ values.

The revised 50% condensation temperatures for Au, As, Cu, Sb, Ga, and Ge at nebular pressures of 10^{-4} and 10^{-6} atm are given in Table 1. As in our previous study, Ge is the most volatile siderophile. The revised Ga 50% condensation temperature is now similar to, or slightly lower than, that of Sb; thus these two elements are the next most volatile elements siderophilic at temperature and redox conditions appropriate to core formation. Our condensation calculations support the idea that the extremely large range in mean group abundances for these elements are a direct reflection of their high volatility in the nebula. In groups having low abundances they seem to have been lost to a large degree during condensation and accretion in the nebula, although we cannot exclude the possibility that some volatile loss occurred in the parent body during the process of melting and core formation.

Fractionation during core solidification

One question remains. If the volatility of Sb is nearly as great as that of Ga, why is it not 'quantised' to nearly the same degree? The answer is to be found in Fig. 3, which shows Ga–Ni, Ge–Ni and Sb–Ni distributions in IIIAB, the largest iron meteorite group. Whereas the Ga and Ge maximum/minimum ratios are 1.3 and 1.6, respectively, that for Sb is about 5. As discussed by Scott¹² and Wasson¹³, there is little doubt that the element–Ni patterns in IIIAB formed by fractional crystallisation of a molten core. During fractional crystallisation elements having solid/liquid distribution coefficients near unity undergo minor fractionations, whereas those having more extreme distribution coefficients undergo large fractionations. This statement

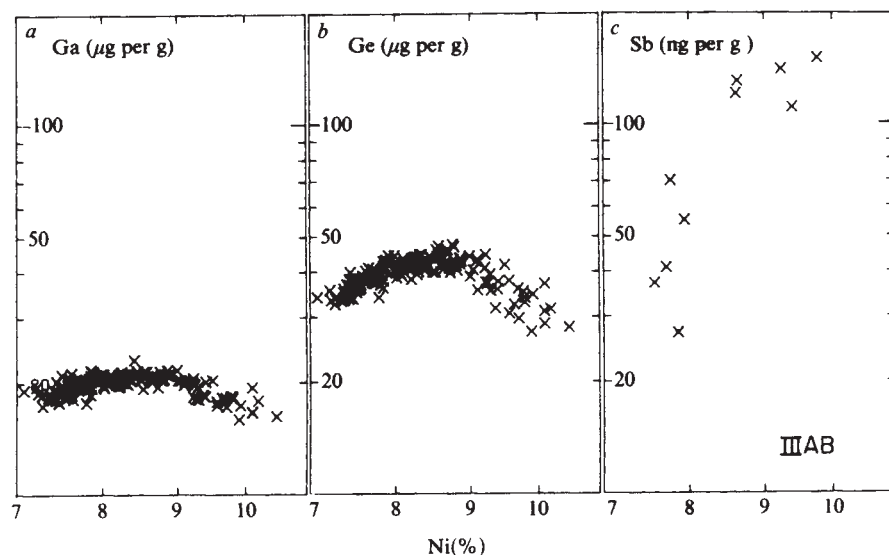


Fig. 3 A detailed comparison of the Ga–Ni (a), Ge–Ni (b) and Sb–Ni (c) distributions in group IIIAB. The small range of Ga and Ge produced during fractional crystallisation results from solid/liquid distribution coefficient near unity (slightly below 1 at low Ni, above 1 at high Ni concentrations). The larger Sb range (factor of 5) reflects a distribution coefficient distinctly different from unity (the estimated value is 0.44).

remains correct even if partial melting or fractional melting were responsible for the observed fractionations.

The distribution coefficient of Ni seems to have remained constant at ~ 0.90 during the fractional crystallisation of most iron meteorite cores^{12,22}. Given this value one can calculate the distribution coefficients of elements linearly related to Ni on log-log plots. For example, the negative linear correlation of Ir and Ni in IIIAB leads to a distribution coefficient of 4.1 and a remarkable maximum/minimum ratio of 2,000. The positive linear correlation between Sb and Ni (Fig. 3) leads to a distribution coefficient of 0.44 and a maximum/minimum ratio of about 5.

In contrast, Ga and Ge are not linearly related to Ni. At low Ni concentrations the slope is positive indicating a distribution coefficient below 1 and at high concentrations it is negative indicating a distribution coefficient above 1. The iron meteorite groups originating in cores show positive Ga- and Ge-Ni correlations at low Ni concentrations, and groups IIAB and IVA

also show the shift to negative slope at high Ni concentrations. In all 11 cases the maximum/minimum ratios are <2 , indicating distribution coefficients near 1. The core-origin group other than IIIAB in which the Sb-Ni distribution is reasonably well defined is IIAB: its Sb range is a factor of 3. Fragmentary data for other groups indicate that ranges of 3–5 are typical for Sb (ref. 41).

Conclusion

The quantisation of Ga and Ge that is so important for the classification of iron meteorites resulted from the fortuitous combination of high nebular volatility, homogenisation during core formation, and low degrees of fractionation during fractional crystallisation of the solidifying cores.

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Thionine coated electrode for photogalvanic cells

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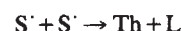
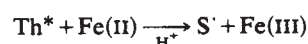
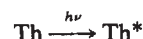
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Russell G. Egdell & Antony F. Orchard

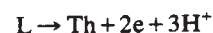
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The successful operation of a photogalvanic cell for solar energy conversion requires that the illuminated electrode should discriminate between the two redox couples in solution. In the iron–thionine system the electrode must oxidise photogenerated leucothionine but not reduce the photo-generated Fe(III). Modified electrodes with coatings of thionine of up to 20 monolayers can be prepared on Pt and SnO₂. Electrode kinetic results for the Fe(III) and thionine systems show that this modified electrode is suitable for the iron–thionine photogalvanic cell.

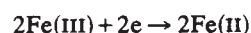
THE criteria required for a successful photogalvanic cell for solar energy conversion has been discussed elsewhere¹. The reaction scheme of the iron–thionine system for such a cell works as follows:



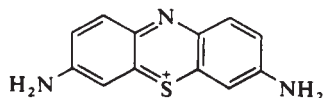
Illuminated electrode



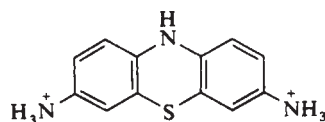
Dark electrode



where Th is



L is



and S' is the semithionine radical.

To obtain power from the cell the illuminated electrode must be able to discriminate between the photogenerated leucothionine (L) and Fe(III)^{1,5}. If the electrode does not discriminate, then addition of the electrode reactions in the reaction scheme shows that the electrode merely catalyses the back reaction of photogenerated products into the original reactants:



The illuminated electrode must remove one of the photo-generated products, in this case L, and force the other, Fe(III), to diffuse across the cell and react on the dark electrode. Note that for an efficient photogalvanic cell the electrode kinetics of the two electrodes must be different⁵. If the Fe(III) reaction is blocked on the dark electrode, as well as the illuminated electrode, then the cell operates only as a concentration cell and produces insufficient voltage of $\sim RT/F$ (ref. 6). We describe here how to modify the illuminated electrode by coating it with thionine itself. The modified electrode then discriminates between the photogenerated L and Fe(III). A photogalvanic cell with a modified and an unmodified SnO_2 electrode will then possess the necessary differential electrode kinetics⁷. The properties of the modified thionine electrode are also interesting in that the electrode discriminates between the organic and inorganic couples studied. The reason for this discrimination will be discussed.

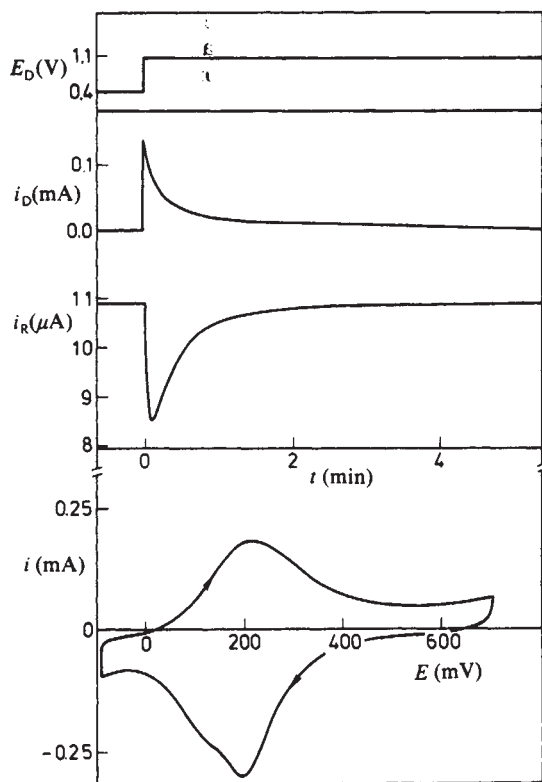


Fig. 1 Ring disk study of coating process and cyclic voltammogram of resulting thionine coated Pt electrode in 0.05 M H_2SO_4 containing no thionine. E_D is the disk potential, i_D the disk current and i_R the ring current.

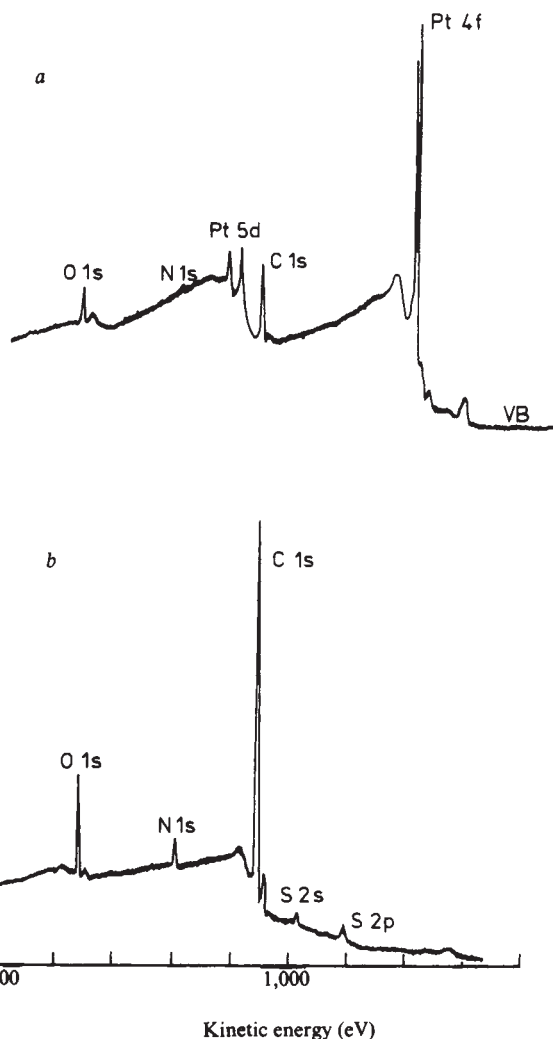


Fig. 2 Typical XPS spectra. *a*, Pt electrode that has been exposed to thionine solution at 0.4 V. The principal feature is the Pt 4f signal. *b*, Pt electrode coated at 1.1 V. The Pt 4f signal is suppressed; C, N and S corresponding to thionine are found.

Experimental methods

All the electrochemical experiments were carried out at 25 °C and all potentials reported with respect to the saturated calomel electrode. Rotating disk and ring-disk studies were performed using an Oxford Electrodes motor controller, rotating assembly and control electronics. Antimony doped SnO_2 electrodes were prepared by spraying a solution of 10 cm³ SnCl_4 and 0.6 cm³ SbCl_3 added to 20 cm³ ethyl acetate for 3 s on to the end of a quartz rod heated to 1,000 °C and rotating at 5 Hz. Spectra were recorded on a Unicam SP 600 recording spectrophotometer. XPS spectra were measured on an AEI ES 200 B photoelectron spectrometer using $\text{MgK}\alpha$ exciting radiation; the base pressure was $\sim 10^{-9}$ torr. The conductance of the thionine coating was measured in the d.c. mode after vapour deposition of a Au contact.

Results and discussion

Preparation and properties of modified electrodes: both Pt and SnO_2 electrodes can be coated with a stable layer of thionine by holding the electrode at 1.1–1.5 V for several minutes in a solution containing thionine. Figure 1 shows a ring-disk experiment which monitors the consumption of thionine during the coating process. The thionine is coated on to the disk electrode and this process is monitored by the downstream ring electrode. From the decrease in the transport limited current for thionine at the ring electrode we can calculate from $N^{-1} \int \Delta i_R dt$

the amount of thionine consumed on the disk where N is the collection efficiency^{8,9}. When the modified electrode is removed from the thionine solution, washed and placed in background electrolyte (such as 50 mM H_2SO_4) containing no thionine, cyclic voltamograms as shown in Fig. 1 are obtained. The peak height of these voltamograms varies linearly with sweep rate as expected for surface bound species. From the charge passed per cycle we can also calculate the amount of thionine in the layer. The coverages found from cyclic voltammetry agree with those from the ring-disk experiment but are usually about 20% lower. Coatings of up to 20 monolayers can be obtained and these coatings are stable in background electrolyte for at least several months. XPS studies, see Fig. 2, of a coated electrode show identical peaks to those of thionine and the Pt 4f peak is completely absent¹⁰. The cyclic voltamograms before and after the XPS experiment show that exposure to high vacuum for several hours only reduces the coverage by about 10%.

Compared with thionine in solution, the visible spectrum of a coated SnO_2 electrode on a quartz plate shows a broadened peak at much the same wavelength ($\lambda_{\text{max}}/\text{nm} \approx 600$) (ref. 10). At reducing potentials this peak disappears. As shown in Fig. 3 the proportion of thionine, f , and leucothionine in the layer obeys the Nernst equation:

$$\frac{RT}{nF} \ln \frac{f}{1-f} = E - E'_c \quad (1)$$

We find $n = 2$ and $E'_c/\text{mV} = 180$. This value is similar to that of thionine in our solution ($E'/\text{mV} = 208$). The resistance of 1 cm^2 of a layer coated at 1.1 V containing 6 nmol cm^{-2} was found to be 60Ω . From these results we conclude: first, the layer consists of thionine or leucothionine depending on the electrode potential; second, the layer is remarkably stable and coherent, it cannot be washed off and the disappearance of the Pt signal in the XPE spectrum is particularly noteworthy; third, for current densities $< 150 \mu\text{A cm}^{-2}$ the voltage drop across the layer is $< 10 \text{ mV}$ and can be neglected.

Thionine: We now examine the electrode kinetics of various redox couples on the thionine coated electrode starting with thionine. Figure 4 shows typical current-voltage curves for the reduction of thionine on a clean and a thionine coated Pt electrode. On the clean electrode the system is reversible. On the coated electrode the system is nearly reversible. Analysis of the current-voltage curves shows that the electrochemical rate constant is about 10 cm ks^{-1} for an electrode coated for 15 min at 1.1 V. Longer coating times or higher coating voltages result

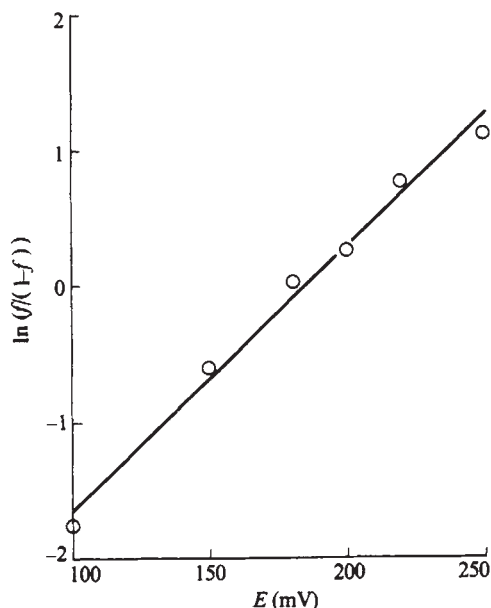


Fig. 3 Plot of Nernst equation (1) describing the bleaching of the thionine layer.

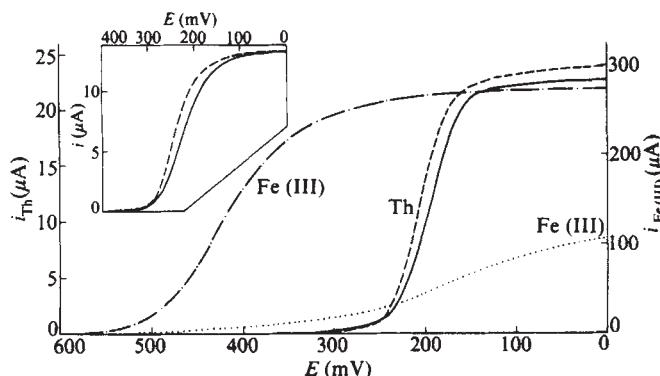


Fig. 4 Typical current-voltage curves for thionine (Th) and Fe(III) on an uncoated (---) and a thionine coated Pt electrode (—). The inset shows similar current-voltage curves for disulphonated thionine.

in electrodes with lower electrochemical rate constants. For instance after coating for 1 h at 1.4 V the rate constant is about 1 cm ks^{-1} . Thus by choosing the coating voltage and time, the performance of the electrode can be controlled. We conclude first that on the coated electrode there is little difference between Pt and SnO_2 as the substrate surface. Second that the electrochemical rate constant is decreased for electrodes coated at higher voltages and for longer times. In particular there is a significant reduction in rate constant for electrodes coated at 1.4 V or above for periods longer than one hour.

Disulphonated thionine: photogalvanic cells have been extremely inefficient for the conversion of solar energy¹¹ because thionine is not sufficiently soluble to absorb the solar radiation close enough to the illuminated electrode⁴. We have recently synthesised 3,6-disulphonated thionine. The solubility is increased so that solutions $\sim 10^{-1} \text{ M}$ can be prepared and the visible spectrum shows no evidence for dimer formation. The disulphonated thionine cannot be coated onto the electrode in the same way as thionine. However, as shown in Fig. 4, the electrode kinetics of disulphonated thionine are very similar to those of thionine. Hence the thionine coated electrode can be used in a photogalvanic cell that operates with the more soluble disulphonated thionine.

Quinone: we investigated the reduction of quinone on the thionine coated electrode and found that the coating had practically no effect. A clean electrode and an electrode coated for 15 min at 1.4 V gave the same rate constant of 0.2 cm ks^{-1} . Hence thionine coating makes little difference to this typical organic redox system.

Fe(III): turning to inorganic couples, we find that the thionine coating makes a very significant difference to the electrode kinetics. We start with the reduction of Fe(III) , which is the reaction that we wish to block in the photogalvanic cell.

Typical current-voltage curves for the reduction of Fe(III) are shown in Fig. 4. The coating produces first a shift of some 200 mV in the half wave potential and second the limiting current of the shifted wave is very much less than that on the untreated electrode. The variation of the limiting currents, i , of the shifted waves with rotation speed, W , are plotted in Fig. 5 according to the Koutecky Levich¹² equation:

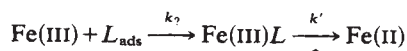
$$\frac{nAF[\text{Fe(III)}]}{i} = \frac{0.65D^{-2/3}\nu^{1/6}}{W^{1/2}} + \frac{1}{k_t} \quad (2)$$

where n is the number of electrons transferred; A , the electrode area; F , the Faraday constant; D , the diffusion coefficient of the electroactive species; ν , the kinematic viscosity; and k_t , the heterogeneous rate constant for the additional rate limiting step.

In equation (2) the term in the rotation speed, $W^{1/2}$, dominates the right hand side when the limiting current is controlled by mass transport. On the other hand the intercept of Fig. 5 gives the term in k_t , which corresponds to the control of the

limiting current by a kinetic process at the electrode which is independent of potential. Analysis of the current-voltage curves shows: first, that the coating has reduced the electrochemical rate constant by 10^{-2} ; second that the transfer coefficient α is still $\frac{1}{2}$; and third that the limiting current is controlled by a second kinetic process described by k_7 .

A reaction scheme of the following type would explain the data,



where k_7 describes the formation of a complex between Fe(III) and coated leucothionine while k' describes an electron transfer with $\alpha \approx \frac{1}{2}$. The fact that $\alpha \approx \frac{1}{2}$ suggests that the potential dependent rate constant is describing an electron transfer, even though the rate is much slower on a coated electrode compared to a clean electrode. The conductivity of the thionine film means that there cannot be large potential differences within the film and hence the main potential drop occurs at the thionine/water interface. We find that although Fe(III) has a different electrode kinetics on clean Pt (fast) and clean SnO_2 (slow), there is not much difference once the electrodes are coated. These results will be discussed further below.

Fe(CN) $_6^{4-}$: results for the oxidation of Fe(CN) $_6^{4-}$ in 0.4 M K_2SO_4 at pH 7 are similar to those for Fe(III). On a clean Pt electrode as expected the couple is reversible but on a coated electrode the wave is irreversible and again there is a second step. For an electrode coated at 1.1 V for 15 min we find $k'_0/\text{cm ks}^{-1} \approx 0.6$ and $k_7/\text{cm ks}^{-1} \approx 2.2$. It is very striking how the thionine coat has blocked this normally rapid electron transfer.

Ru(bpy) $_3^{3+}$: even more dramatic are the results for the reduction of Ru(bpy) $_3^{3+}$ in 0.5 M H_2SO_4 (where bpy is 2,2' bipyridine). Results on a coated electrode are shown in Fig. 6. On a clean electrode Ru(bpy) $_3^{3+}$ is reduced at potentials < 1.0 V, in a reversible wave. The thermodynamics for the reduction are favourable and the kinetics are rapid. However, on the coated electrode the transport limited current is not reached until 0.30 V. The current-voltage curve shows that there are two parallel processes. For potentials > 0.40 V we find a small current which increases gradually as the potential decreases. A reasonably

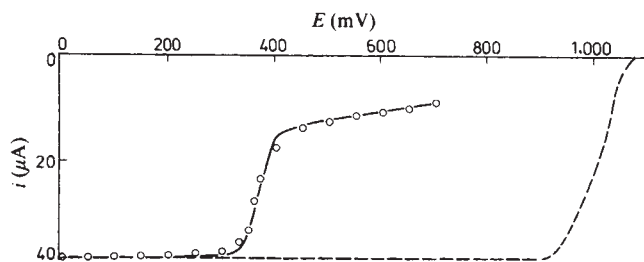
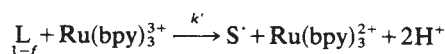
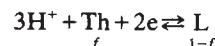


Fig. 6 Current-voltage curves for reduction of Ru(bpy) $_3^{3+}$ on a clean (---) and a coated (O) Pt electrode. The solid line is the calculated curve for the two parallel processes discussed in the text.

straight Tafel plot is obtained with a low value of $\alpha = 0.05$. At 0.4 V a second process takes over and the current rises rapidly to its transport limited value. Analysis of this part of the current-voltage curve gives $\alpha \approx 2$. This high value of α cannot be caused by driving the electron transfer for the reduction of Ru(III). For that reaction we would expect (as was found for Fe(III)) that $\alpha = \frac{1}{2}$, and in any case α must be < 1 . We propose that the second process is the reaction of Ru(III) with L formed on the outside of the layer:



The very small fraction of L ($\sim 2 \times 10^{-3}$) present when the second process takes over can be estimated from the Nernst equation (1) and the value of E'_c for 0.5 M H_2SO_4 where $E'_c/V = 0.270$. Figure 3 shows that the oxidation state of the thionine layer obeys the Nernst equation, and so we can calculate the current-voltage curve in Fig. 6 using the parameters derived from the analysis. Although our model for the second process fits the data very well, we do not yet have a detailed model for the slow process occurring at potentials > 0.4 V. The very low value of α means that the process is not a straightforward electron transfer. This process may be similar to the k_7 step in the reduction of Fe(III). The potential range over which we can observe the k_7 process for Fe(III) is too restricted to be certain if k_7 depends slightly on potential or not.

Ce(IV): the reduction of Ce(IV) resembles that of Ru(bpy) $_3^{3+}$. Again two processes are evident. Following the same analysis as for Ru(bpy) $_3^{3+}$ we find that the steep part gives a curved Tafel plot with α varying between 1.4 and 2.0. The fact that these values are > 1 suggests that we must again be seeing a reaction between L in the coat and Ce(IV).

Effect of coating voltages and coating times: Our interest in photogalvanic cells has led us to investigate the thionine and Fe(III) systems in more detail. We find that there is some reduction in the kinetic parameters the higher the coating voltage and the longer the coating time. We have found that there is a good correlation between the electrochemical rate constants for thionine or for Fe(III) and k_7 . As these rate constants describe different processes we assume that the correlations arise through a general effect of the higher voltage or coating time on the coat itself. This affects all three processes, the reduction of thionine, the electrochemical step and the k_7 step in the reduction of Fe(III), to much the same extent. The higher coating voltages and coating times may cause this, for instance, by blocking off active sites on the surface.

Inorganic electron transfers: While the coat seems to have no drastic effect on the reduction of thionines or of quinone, single electron transfers to inorganic ions which normally proceed by outer sphere mechanisms are in all cases blocked by the coat. This is probably because the thickness of the coat prevents the direct tunnelling of electrons from the metal or SnO_2 to the solvated inorganic ion. For strong oxidising agents like Ru(III)

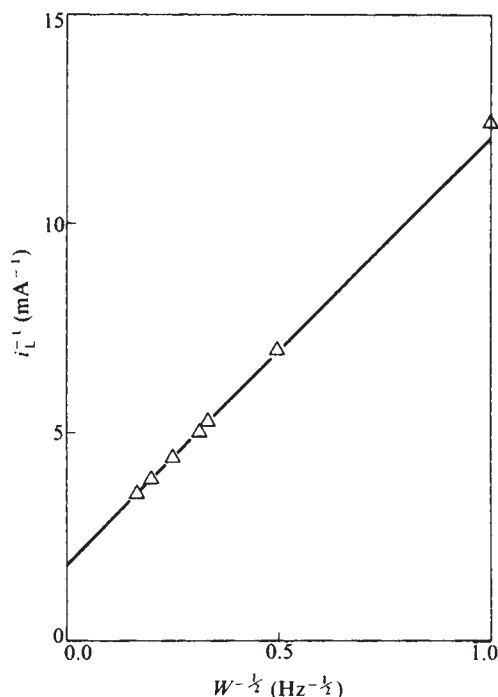


Fig. 5 Koutecky Levich plot, equation (2), for reduction of Fe(III) at thionine coated Pt electrode.

and Ce(IV) the main path for reaction is apparently the oxidation of leucothionine on the outside of the coat. For Fe(III), because there is not such a strong thermodynamic driving force, the reaction between Fe(III) and L is 7 orders of magnitude slower than diffusion control. Hence we apparently have a two step mechanism of which one step is an electron transfer and we suggest that the other is the formation of a 'complex' between Fe(III) and leucothionine at the surface. Such complexes have been found in homogeneous reactions between Fe(III) and L (ref. 13). Note that the half wave potential for the reduction of Fe(III) is close to that of E' for thionine suggesting that the conversion of thionine to leucothionine in the coat may well be necessary for the reaction.

Implications for photogalvanic cells: Finally we return to the question of the selectivity of these electrodes for use in

photogalvanic cells. Figure 4 shows that the thionine coated electrode does have the required selectivity. The coating leaves the electrode kinetics of the thionine couple sufficiently rapid to handle the photogenerated leucothionine⁴ but it blocks the reduction of Fe(III). Thus an illuminated electrode working at a potential where it is oxidising leucothionine will only have a small current caused by the simultaneous reduction of Fe(III). Hence we conclude that the thionine coated electrode is an ideal illuminated electrode for the iron-thionine photogalvanic cell. The fact that it can be made so easily and that it is relatively stable are added advantages.

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Identification of the protein encoded by the transposable element Tn3 which is required for its transposition

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Protein products have now been identified which account for the entire coding capacity of the transposable element Tn3. Mutations in Tn3 have allowed us to map the genes encoding each of these peptides and to identify their role in transposition. We have found that only a single Tn3-encoded peptide is required for transposition. Expression of this peptide is repressed by the product of a second gene, which is itself autogenously regulated.

PLASMID-MEDIATED antibiotic resistance in bacteria has often been shown to reside on discrete DNA segments capable of transposition to other replicons in the cell (for review see refs 1-5). This event occurs in the absence of *recA*-mediated recombination and does not require extensive regions of homology. One such transposable element, Tn3 (refs 6-8) confers resistance to ampicillin by encoding for the production of the penicillin hydrolysing enzyme β -lactamase. This transposon is a particularly useful focus of study for the examination of the transposition process as a molecular event.

Analysis of mutants of the Tn3 element^{9,10} has resulted in a relatively simple map of the genetic organisation of the Tn3 functions and structures necessary for its transposition and those related to the regulation of transposition frequency (Fig. 1). The element itself is about 5,000 base pairs in length. The 38 base pairs at each end are inverted and repeated sequences (IR)¹¹. Mutations affecting either of the IR result in a transposition-

deficient phenotype, and at least the left IR is required in *cis* (ref. 10). (The element will always be discussed in the orientation shown in Fig. 1.) The structural gene for the β -lactamase enzyme (*bla*) maps to the far right end of the transposon^{9,10} (Fig. 1). Adjacent to the *bla* gene and bisected by the *Bam*HI restriction site, is the part of the transposon which we refer to as the 'regulatory region'. This region is known to consist of at least one gene, which we designate *mpR*, which encodes a *trans*-acting 19,000-molecular weight (MW) peptide that represses the level of Tn3 transposition^{9,10,12,13}. Adjacent to the regulatory region and extending to the left IR is a 3000-base pair stretch of DNA which encodes one or more *trans*-acting functions necessary for Tn3 transposition^{9,10,12}. For convenience, we shall refer to this segment of the element as the 'transposase' region. This terminology, however, is only descriptive of the phenotype of mutations which saturate this region of the Tn3 map; it is not meant to imply any particular enzymatic activity for the functions encoded by this region.

The genetic map of Tn3 had been formulated from the analysis of two sets of Tn3 mutants^{9,10}. Both were the result of *in vitro* manipulation of the plasmid RSF1050, a small derivative of ColE1 into which Tn3 had been transposed. The first set of RSF1050 mutants contained various-sized deletions having one or both endpoints within the transposon⁹. The newer collection was constructed by random *in vitro* insertion of an oligonucleotide containing an *Eco*RI restriction site into the Tn3 element¹⁰. We will refer to this collection of mutants as 'EcoRI-insertion mutants'. Many of the *Eco*RI-insertion mutants have now been sequenced (F. Heffron *et al.*, in preparation). It was found that, in addition to the insertion of the *Eco*RI site oligonucleotide, a small deletion or duplication of Tn3 sequences occurred at the site of the insertion. However, none of these was greater than 50

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base pairs. The result is that many of the mutations shift the translational reading frame distal to the mutation. These would, therefore, be expected to have the same utility as frameshift mutations for mapping location and direction of gene transcription. Other *EcoRI*-insertion mutations have been found to retain the original reading frame and so will be expected simply to result in the addition of a few amino acids to the translation product at the point of the mutation.

These mutants were used to map the *tnpR* gene and study its regulation of transposition frequency. They have also allowed us to identify the peptide encoded by the 'transposase' region of the element.

Genetic analysis identifies only a single Tn3 gene necessary for its transposition

Previous analyses of Tn3 mutants have shown that all of the recessive transposition-deficient (Tnp^-) mutants are clustered in the transposase region of the element^{9,10}. To establish a genetic basis with which to approach the biochemistry of transposition, we wished to determine the number of functions encoded by this region which are required for transposition to occur. Therefore, we initially attempted a complementation analysis of these Tnp^- mutants.

To use the existing Tn3 mutants, which were all isolated on the plasmid RSF1050, it was necessary to circumvent the problem of plasmid incompatibility when testing pairs of these mutants for complementation. Therefore, a number of Tnp^- deletion or insertion mutants of Tn3 were transposed by complementation by wild-type Tn3 on to a plasmid compatible with RSF1050; either JCFL4 (*F'* *lac tra_{am}K4*)¹⁴, or a non-colicinogenic derivative of the plasmid ColK (provided by D. Sherratt). These plasmids were then introduced into *recA*⁻ cells containing a conjugative plasmid and representative RSF1050 Tnp^- mutants. Strains containing each of the combination of Tn3 mutants were tested for complementary transposition using the mating assay described previously^{9,12}. In this manner, 5 deletion and 16 *EcoRI*-insertion mutants spanning the transposase region were tested in pairwise combinations for complementation. Data for representative Tnp^- mutants are shown in Table 1. Although the assay was sensitive

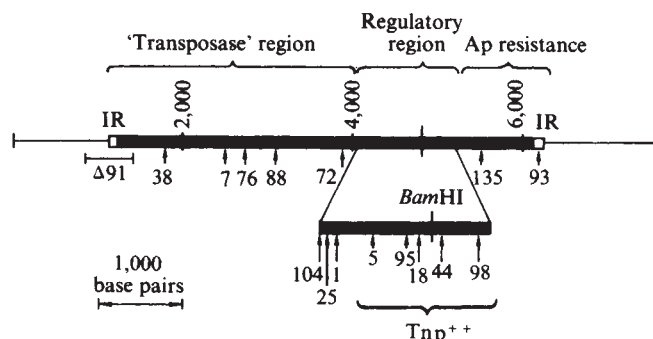


Fig. 1 Map of the Tn3-containing plasmid RSF1050^{9,10}. The plasmid is shown as a linear molecule after cleavage at the *EcoRI* site in the ColEI portion of the molecule. The Tn3 element is indicated by the bold line; ColEI-derived sequences are denoted by the thin line. The inverted and repeated termini of the element, labelled IR, are denoted by an open box. The Tn3 element is divided into three sections based on the phenotype of mutations which map there, as explained in the text. Arrows (↑) denote location of *EcoRI*-insertion mutations¹⁰; each is identified by its pFH number. pFH7, pFH76 and pFH88 are Tnp^- ; those mapping to the regulatory region are Tnp^{++} ; pFH135 has lost the ability to confer resistance to ampicillin; pFH93 is a *cis*-dominant Tnp^- mutation affecting the right IR, which is able to complement mutations in the 'transposase' region; mutation $\Delta 91$ is a deletion which removes about 300 base pairs of Tn3, including the left IR (F. Heffron *et al.*, in preparation).

enough to detect transposition at a frequency of at least 1% of the wild type, these experiments failed to detect complementation between any of the pairs of mutants tested. These results indicate that Tn3 encodes only a single *trans*-acting function in the transposase region, although other explanations could not be eliminated by these experiments.

Identification of Tn3-encoded peptides in minicells

We next attempted to confirm our genetic evidence for a single function encoded by the Tn3 transposase region by visualising its protein products directly. Dougan *et al.*¹³ have reported the

Table 1 Complementation of Tn3 mutants

	JCFL4 derivative*	RSF1050 derivative					Comments
		A RSF1050 ⁺	B pFH72	C pFH88	D pFH7	E pFH38	
1	JCFL4	(—)	0	0	0	0	Negative control. The level of transportation of Tn3 from the Tnp^- mutants of RSF1050 was below the limit of detection (0.5×10^{-6} or a normalised value of 0.004)
2	JCFL4::Tn3-135 ⁺	1.0	0.3	0.1	0.4	0.1	Positive control. Tnp^- mutants were complemented <i>in trans</i> by a transposition proficient Tn3 element on JCFL4 ⁺
3	JCFL4::Tn3-72	1.0	0.0009	0.006	0.001	0.003	The Ap ^r transconjugants reported in columns B-E had received the JCFL4 derivative and represent reversion or suppression of the <i>traK4</i> mutation on the <i>F'</i> <i>lac</i> . No transposition on to R388 was detected
4	JCFL4::Tn3-38	1.0	0.002	0.001	0.007	0.003	

Complementation experiments were attempted using strain M1653 (*recA*⁻, *Su*⁰) containing the conjugative plasmid R388 (trimethoprim and sulphonamide resistant) plus representative Tnp^- mutants of RSF1050. The plasmid JCFL4 (*F'**lac traK4*) into which representative Tnp^- mutants of Tn3 had been transposed was then introduced into these strains. JCFL4 was not transmissible from the *Su*⁰ strain, M1653. Therefore, transposition (by complementation *in trans*) of either of the mutant Tn3 elements into the co-resident conjugative R388 plasmid could be measured by selecting for the transfer of Ap^r into a suitable recipient in a 4 h mating. The frequency of transposition was: no. of Ap^r transconjugants/total no. of transconjugants. Representative data are presented for four Tnp^- mutants of RSF1050 (columns A-E) and two Tnp^- mutants of Tn3 on JCFL4 (lines 3 and 4). The values entered into the table were normalised to the frequency of transposition of wild-type Tn3 from RSF1050 (1.3×10^{-3}) which was given a value of 1.0.

* Locations of the respective mutations in Tn3 are shown in Fig. 1.

† Tn3-135 (ref. 9) is a Tn3 derivative which has a determinant encoding tetracycline resistance cloned into and inactivating the ampicillin resistance gene. Transposition functions have not been detectably altered. Use of this element made it possible to directly select for the complemented transposition of the co-resident Tnp^- (Ap^r) element on RSF1050.

use of *Escherichia coli* minicells¹⁵ containing RSF1050 or representative RSF1050 deletion mutants to identify polypeptides encoded by defined regions of the Tn3 element. These investigators reported, however, that no protein encoded by the transposase region of the element was synthesised in detectable amounts by minicells. The functions encoded by this region are presumably repressed by the wild-type element^{10,12}.

E. coli minicells were also used here, but advantage was made of several recently isolated transposition-proficient mutants of Tn3 which had lesions confined to the regulatory region of the element¹⁰. These mutations resulted in a 10–50-fold increased frequency of transposition of the mutant element, and were recessive to wild-type Tn3 for this behaviour (ref. 10, R. E. G., unpublished observation). The phenotype of these mutants, designated Tnp⁺, suggests that they possess mutations which allow increased expression of the function(s) encoded by the transposase region.

Five of the Tnp⁺ mutants of RSF1050 used in this study were from the collection of *Eco*RI-insertion mutants isolated by Heffron *et al.*¹⁰ (see Fig. 1). The remaining Tnp⁺ mutant was prepared by enzymatic manipulation of the *Bam*HI restriction site of Tn3. *Bam*HI cleaved RSF1050 only once, at a site in the Tn3 regulatory region, generating a linear molecule with single-stranded 5' protruding ends. These ends were made double stranded by polymerising the complementary strand with T4 DNA polymerase. This manipulation introduced a 4-base pair duplication at the *Bam*HI site: G¹GATCC to GGATCGATCC. Consequently, a frameshift mutation was introduced into the gene encoded at that point. As the mutant

sequence was no longer cleaved by *Bam*HI, the mutant element will be referred to as a *Bam*HI⁰ mutant.

We have examined the Tn3 specific polypeptides synthesised in *E. coli* minicells carrying each of the RSF1050 Tnp⁺ mutants (Fig. 2). Peptides encoded by wild-type Tn3 can be identified by comparing lane a, pMB8, with lane b, RSF1050 (pMB::Tn3). The β -lactamase gene product, which is known to undergo post-translational processing^{16,17} resolves into three discrete bands with our electrophoresis conditions (also see ref. 13). Minicells carrying wild-type Tn3 (lane b) also synthesise a 19,000-MW peptide. This peptide was shown by Dougan *et al.*¹³ to be encoded by the Tn3 regulatory region by the gene we designate here as *tnpR*. They also observed the correlation that Tn3 mutants having the phenotype of increased transposition frequency failed to produce this 19,000-MW peptide.

In contrast to the wild-type element, the 19,000-MW peptide is not synthesised in minicells carrying any of the Tnp⁺ mutants (Fig. 2, lanes c–h). Minicells containing pFH5, pFH95, a *Bam*HI⁰ mutant of Tn3, or pFH44 (Fig. 2, lanes c–f) each produced an additional prominent peptide which we interpret to be truncated forms of the 19,000-MW *tnpR* gene product. The truncated peptide encoded by pFH5 has eluted off the gel, but those encoded by pFH95, the *Bam*HI⁰ mutant, and pFH44 migrated with apparent molecular weights of 11,500, 12,500 and 15,500, respectively. The peptide from the *Bam*HI⁰ mutant co-migrated with a peptide encoded by the pMB8 portion of RSF1050 in these electrophoretic conditions. The size of the truncated peptide encoded by each of the mutants is related to the map position of the mutation in such a way as to suggest that transcription of *tnpR* proceeds rightward and that the initiation of translation corresponds to the 4,200 base pair coordinate of RSF1050 (Fig. 1). This is between the mutation in pFH1 and pFH5, supporting the observation that mutation 1 does not lie in the *tnpR* gene itself (see below).

Minicells containing the Tnp⁺ mutants pFH18 and pFH98 also fail to synthesise a 19,000-MW peptide (Fig. 2, lanes g, h). However, each encodes a new peptide slightly larger than this. These peptides have been shown to be highly related to the wild-type protein by comparison of the fragments generated by partial chymotrypsin or *Staphylococcus aureus* V8 protease cleavage¹⁸ of each of the respective peptides (data not shown). The nucleotide sequence surrounding mutations 18 and 98 have been determined (F. Heffron *et al.*, in preparation). Mutation 18 was found to preserve the transcriptional reading frame of the distal portion of the *tnpR* gene, but had an insertion at the site of the mutation sufficiently large to account for the synthesis of the observed peptide. A somewhat similar explanation accounts for the observed peptide encoded by pFH98.

Dougan *et al.*¹³ have previously observed that truncated forms of the 19,000-MW peptide from *tnpR* mutants of the Tn3 were overproduced in minicells. Results shown in Fig. 2 tend to corroborate that observation, at least for pFH44, pFH18, pFH98 and the *Bam*HI⁰ mutant. As measured by the density of the band image on fluorograph negatives, considerably more radioactivity was incorporated into the mutant *tnpR* products than into the wild-type 19,000-MW peptide. It seems, therefore, that the *tnpR* gene product represses the level of its own expression, as well as repressing the level of transposition.

Interestingly, the fluorograph in Fig. 2 also clearly shows that minicells containing any of the Tn3 mutants which affect the 19,000-MW peptide (lanes c–h) synthesise a previously undetected high molecular weight peptide. This peptide migrates between β -galactosidase (MW 135,000) and phosphorylase *b* (MW 100,000) in 7.5% SDS polyacrylamide gels¹⁹ and has an apparent molecular weight of 120,000. Disregarding the possibility of overlapping genes, the transposase region of the element is just large enough to encode a 120,000-MW protein, and is the only such stretch of DNA on the transposon for which no other protein product has been identified.

We have examined the effect of Tnp⁺ mutations on the synthesis of this peptide to determine if it was, in fact, the product of the transposase region. We have already shown that

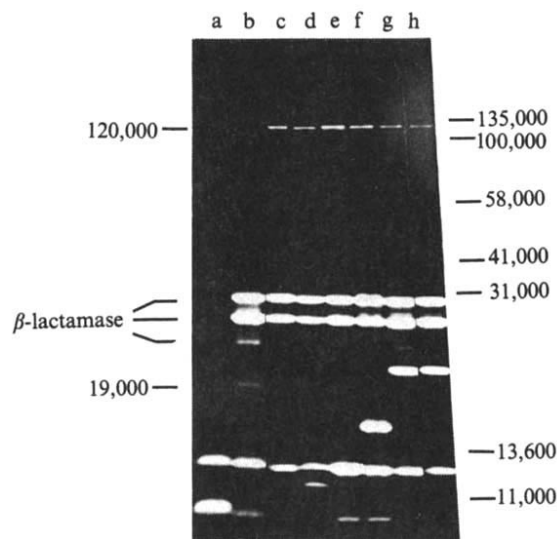


Fig. 2 Identification of Tn3 encoded peptides. Minicells were purified²² from cultures of *E. coli* DS410²³ containing the plasmid RSF1050 or Tnp⁺ mutants of RSF1050. Proteins were labelled in M9 minimal media with 30 μ Ci ml⁻¹ of ³⁵S-methionine for 45 min. Minicells were collected and lysed by boiling in 80 μ l of cracking buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β -mercaptoethanol, 5% glycerol). The proteins were precipitated by addition of 1.2 ml of acetone for 20 min at room temperature. The precipitate was pelleted and resuspended in final sample buffer (0.0626M Tris, pH 6.8, 3% SDS, 5% β -mercaptoethanol, 10% glycerol), and boiled 3 min. Approximately, 60,000 c.p.m. were applied to each well of a 10–18% gradient SDS polyacrylamide gel¹⁹, and electrophoresed at 130 V until the dye reached the bottom of the gel. Gels were prepared for fluorography as described²⁴ and exposed for 18 h at -70°C . Samples were prepared from minicells carrying the following plasmids: Lane a, pMB8, which is the ColEI derivative into which Tn3 was transposed to make the plasmid RSF1050; lane b, RSF1050, (pMB8::Tn3, wild type); lanes c–h Tnp⁺ mutants pFH5, pFH95, a *Bam*HI⁰ mutant of RSF1050, pFH44, pFH18 and pFH98, respectively. Location of each mutation is shown in Fig. 1. Migration of molecular weight standards is shown on the right margin. Standards were: *E. coli* β -galactosidase, MW 135,000; phosphorylase *b* (rabbit muscle), MW 100,000; catalase (beef liver), MW 58,500; alcohol dehydrogenase (horse liver), MW 41,000; DNase 1 (bovine pancreas), MW 31,000; RNase A (bovine pancreas), MW 13,600; cholera toxin B subunit, MW 11,000. The 120,000 and 19,000-MW peptides from Tn3 and the three forms of the β -lactamase gene product are identified in the left margin.

this protein is only made in detectable quantities by transposons which have mutations in the *tnpR* gene. Therefore, representative *Tnp⁻* deletion and *EcoRI*-insertion mutants of Tn3 were also made *tnpR⁻* by introducing a 4 base-pair insertion at the *Bam*HI site, as described above. The proteins encoded by these mutant elements were labelled in minicells and separated on SDS polyacrylamide gels (Fig. 3).

As expected of *Bam*HI⁰ mutants, each of the minicell strains produced the characteristic 12,500-MW truncated *tnpR* product. None of these doubly mutant elements directed the synthesis of the 120,000-MW peptide, however (Fig. 3, lanes c-f). Furthermore, only mutations in the transposase region abolished synthesis of this peptide; *Bam*HI⁰ mutants of elements having *EcoRI*-insertion mutations elsewhere in the element, either in the β -lactamase gene (mutant 135) or in the right IR (mutant 93) continued to direct the synthesis of the 120,000-MW peptide (data not shown). These results indicate that the transposase region itself encodes the 120,000-MW peptide expressed in the *tnpR⁻* mutants of Tn3. We have designated the gene encoding this peptide *tnpA*.

The *EcoRI*-insertion mutants used in Fig. 3 were selected because DNA sequencing had shown that they led to a shift in the transcriptional reading frame of the *tnpA* gene (F. Heffron *et al.*, in preparation). Thus, they could be used to determine the location of the ends of the *tnpA* gene and the direction of its transcription. Minicells containing *Bam*HI⁰ mutants of pFH7, pFH76¹⁰, and pFH88 (lanes d-e) synthesised unique 60,000, 52,000 and 37,000-MW peptides, respectively. The molecular weight of these unique peptides correlated with the map position of the mutations in such a way as to suggest that the *tnpA* gene is transcribed leftward on the Tn3 map in Fig. 1. Assuming that an amino acid has an average MW of 110, it was deduced that the beginning of translation of the *tnpA* gene product corresponds approximately to the 4,100 base pair coordinate of RSF1050, which in turn places the distal end of the gene very close to the left IR. The orientation of the *tnpA* gene was also deduced from the effect of the deletion Δ 91 on the synthesis of the 120,000-

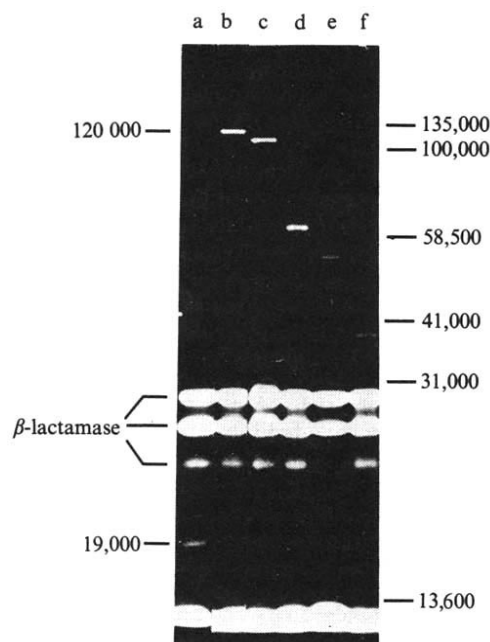


Fig. 3 Peptides synthesised in minicells containing transposition-deficient mutants of Tn3. Minicells were isolated, labelled and prepared for electrophoresis as in Fig. 2. Extracts were electrophoresed through an 11% SDS polyacrylamide gel at 100 V. Lane a, wild-type RSF1050; lane b, *Bam*HI⁰ mutant of RSF1050. Lanes c-f, RSF1050 derivatives which have a *Bam*HI⁰ mutation, as well as a mutation in the 'transposase' region of Tn3. The 'transposase' region mutations are: lane c, deletion 91; lane d, *EcoRI*-insertion mutation 7; lane e, *EcoRI*-insertion mutation 76; lane f, *EcoRI*-insertion mutation 88. Location of these mutations in Tn3 is shown in Fig. 1. Molecular weight standards are those listed in Fig. 2 legend.



Fig. 4 Map Tn3 showing location of its three genes. Arrows indicate direction of transcription. The size of the protein product of each gene is also indicated. Location of the *bla* gene and direction of its transcription are based on Heffron⁹ and Sutcliffe¹⁶. Three peptides are associated with the β -lactamase gene in minicell extracts¹⁴. The largest of these, with MW of 29,000 when compared to our standards, is presumably the unprocessed *bla* gene product^{16,17}.

MW peptide. Δ 91 deletes ~300 base pairs of the left end of Tn3 including the left IR (Fig. 1). This deletion reduced the molecular weight of the peptide from 120,000 to 105,000, presumably by removing the distal portion of the gene. It is interesting to contrast the phenotypes of Tn3 elements having the Δ 91 deletion with that of the *EcoRI*-insertion mutant pFH93. Mutant Δ 91 removes the left IR; mutation 93 interrupts the right IR (Fig. 1). Both are *cis*-dominant *Tnp⁻* mutations, presumably because both the left and right IR are required for transposition. Mutation 93 did not affect the *tnpA* gene product, and was found to complement *tnpA* mutants of Tn3 (data not shown). Deletion mutant Δ 91, however, will not complement over *Tnp⁻* mutants, presumably because of the removal of the distal portion of the *tnpA* gene. We have also examined three additional transposition-proficient *EcoRI*-insertion mutants, pFH104, pFH25 and pFH1, which map just to the left of the *Tnp⁺⁺* mutations (Fig. 1). The peptides synthesised from minicells containing these mutant plasmids appear identical to those encoded by the wild-type element (data not shown). In addition to the β -lactamase peptides, each of the mutant plasmids directs the synthesis the 19,000-MW product of the *tnpR* gene, but not detectable amounts of the *tnpA* gene product. Expression of the 19,000-MW peptide is consistent with the observation that these three mutants retained the ability to repress the frequency of transposition of *Tnp⁺⁺* (that is, *tnpR⁻*) mutants. It also supports our earlier placement of the proximal end of the *tnpR* gene between the mutations in pFH1 and pFH5. However, whereas pFH1 transposed at near wild-type levels, mutants pFH104 and pFH25 each transposed at an elevated frequency, intermediate between wild-type and the *Tnp⁺⁺* mutants, and were apparently repressed by a wild-type element (R.E.G., unpublished observation). The observation that pFH25 and pFH105 transposed at an elevated frequency, even though functional *tnpR* gene product was being produced, was unexpected. We do not believe, however, that these mutants contradict our scheme for the regulation of Tn3 transposition frequency. Indeed, DNA sequence analysis of these mutations (F. Heffron, *et al.*, in preparation) indicated that these mutations actually affect the proximal end of the *tnpA* gene. If this is the case, then the increased transposition frequency may reflect an alteration in the activity of the *tnpA* gene product, rather than in its regulation.

Conclusion

Tn3-specified proteins have now been identified which account for the entire coding capacity of the transposon. Our data indicate the presence of three genes encoded by the element as outlined in Fig. 4. The *bla* gene, encoding the β -lactamase enzyme, is situated at the right end of the element and is transcribed rightwards^{9,16}. The remaining two genes, *tnpA* and *tnpR*, begin near the centre of the transposon and are transcribed in opposite directions. The position of these genes is consistent with the genetic organisation of the element as deduced from the DNA sequence of the element (F. Heffron *et al.*, in preparation).

The *tnpA* gene extends from its initiation near the centre of the element all the way to the left IR. All of the known recessive *Tnp⁻* mutations of Tn3 map in this gene. The existence of only a

single gene encoded by the transposase region is also supported by the observation reported here that recessive *Tnp⁻* mutants appear to fall into a single complementation group. The product of the *tnpA* gene has been identified as a 120,000-MW peptide, which is expressed in detectable quantities by minicells containing only *tnpR⁻* mutants of Tn3. As this peptide is required for transposition, its overproduction by *tnpR⁻* mutants may account for the *Tnp⁺⁺* phenotype of such mutants. Although necessary for transposition, no enzymatic function has yet been attributed to this peptide, and its possible interaction with host proteins has not been established.

The product of the *tnpR* gene is a 19,000-MW peptide. Our data are consistent with a dual regulatory role for this gene product. First, it appears to repress the level of *tnpA* expression.

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Overproduction of the 120,000-MW *tnpA* gene product by *tnpR⁻* mutants presumably accounts for the elevated transposition frequency observed for these mutants. Second, the *tnpR* gene seems to regulate the level of its own expression. The mutant *tnpR* gene products are typically overproduced relative to the wild-type product. Regulatory proteins having such dual roles have also been observed for the *araC* gene²⁰ and *cI* gene of bacteriophage²¹.

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Transposition protein of Tn3: identification and characterisation of an essential repressor-controlled gene product

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Fusions that bring lac gene expression under the control of transcriptional and translational signals within the Tn3 element have been used to identify and characterise a Tn3-encoded 'transposase' (Tnp) peptide of MW 100,000 essential for transposition. The gene specifying this product is regulated by the Tn3 repressor protein and is part of a bidirectional genetic unit that includes the repressor gene.

Tn3 is one of a class of genetic elements capable of transposition between DNA segments having no sequence homology with each other or with the transposable element itself¹. This element encodes functions that affect its own transposition^{2,3} as well as a β -lactamase gene that renders host bacterial cells resistant to the antibiotics penicillin and ampicillin⁴.

Recently, we have used Tn3-*lac* gene fusions to identify and analyse regulatory mechanisms that control the transposition of Tn3 (refs 5-7). Plasmids containing both Tn3 and *lac* were constructed and deletions that fuse the *lac* genes to Tn3 sequences were introduced, bringing expression of *lac* under control of transcriptional and translational signals within the transposable element. Such experiments have shown that a DNA region within Tn3 that is known to influence its transposition frequency^{2,3,8} encodes a peptide of molecular weight 21,000 (21K) that represses transposition of Tn3 and acts at the level of transcription to regulate its own synthesis⁷.

During our studies of the Tn3-encoded repressor gene, we observed a prominent increase in the synthesis of a 100K peptide in Tn3 mutants that fail to synthesise a functional repressor, suggesting that the gene specifying this peptide might also be regulated by the repressor. Here we report the use of *lac*

gene fusions to identify and characterise the gene encoding the 100K peptide. Our findings indicate that this peptide is a repressor-controlled product required for transposition of Tn3 and that its gene is transcribed in a divergent direction from the adjacent repressor gene.

Identification of a Tn3-encoded 100K peptide

Gel electrophoresis (Fig. 1a) of extracts from ³⁵S-methionine-labelled minicells⁹ containing a nonsense (R49) or missense (R102) mutation in the repressor peptide showed a large band having a calculated molecular weight of 100K for both types of repressor-minus mutants; little or no such peptide was seen in extracts of minicells containing the wild-type (repressed) Tn3.

The location and extent of the gene encoding the 100K peptide were identified using the pMC1102 and pMC1104 plasmids (Fig. 2), which carry Tn3 elements that have deleted internal sequences, and the pMC823 plasmid, which has deleted all of Tn3 to the right of the sixth codon of the repressor gene (ref. 7; see also Fig. 4 below). The pMC1102 and 1104 plasmids include the R49 amber mutation in the Tn3 repressor gene. As seen in Fig. 1, pMC1102 ($\Delta BclI$) and pMC1104 ($\Delta PstI$) (Fig. 1b) do not encode the 100K peptide whereas pMC823 does (Fig. 1c). This locates the 100K peptide gene between the left end of Tn3 (IRa) and the beginning of the repressor gene, which is the only region of Tn3 retained on pMC823. From the size of the 100K peptide, it can be calculated that almost this entire segment must be used to encode the peptide (Fig. 2). The orientation of the gene for the 100K peptide can be inferred from the size of the 66K truncated peptide (Fig. 1b) formed by the pMC1104 ($\Delta PstI$) deletion plasmid. The 66K peptide is too large to be encoded entirely to the left of $\Delta PstI$ and too small to

span it in frame. Thus, the NH₂-terminus of the 66K peptide can be assigned to the right of $\Delta PstI$, thus implying a right-to-left orientation for the gene. This assignment is consistent with the absence of a detectable truncated peptide for the pMC1102 ($\Delta BclI$) deletion plasmid which retains only a very small part of the right end of the 100K gene.

Assignment of the gene for the 100K peptide gene to a location at the left end of Tn3 (Fig. 2) exhausts the coding capacity of the transposon, unless overlapping or very small genes are present. Tn3 is thus seen to encode three peptides—a β -lactamase^{4,10}, a self-regulated repressor protein⁷, and the repressible 100K peptide.

The $\Delta PstI$ and $\Delta BclI$ deletions, both of which fail to synthesise the 100K peptide (Fig. 1), do not undergo transposition at a detectable frequency even when coupled to a mutation (R49) in the Tn3 repressor gene that otherwise leads to a 14-fold increase in the frequency of transposition (data not shown). This observation, together with earlier evidence^{2,3} that deletions or insertions in the vicinity of these endonuclease cleavage sites prevent transposition, suggests that the repressor-controlled 100K peptide (hereafter termed 'transposase' or *Tnp* gene product) carries out functions essential to the transposition process.

The effects of repressor on synthesis of the *Tnp* protein are shown in Table 1. As seen in the table, missense and amber mutations in the Tn3 repressor lead to a 10- and 60-fold increase in production of a missense repressor peptide or a nonsense truncated peptide respectively. The greater extent of derepression observed with the nonsense mutation (R49), which yields a truncated repressor peptide⁷, compared with the missense mutation (R102), which results in the formation of a full-length peptide having a presumed amino acid substitution, could be explained if the peptide product of the missense mutant

is partially functional. This interpretation is consistent with the observed fivefold increase of the *Tnp* peptide in the missense mutant versus the eightfold increase of the *Tnp* peptide in the two nonsense mutants examined.

Use of *lac* gene fusions for study of regulation of the *Tnp* protein

Introduction of a promoter-less *lac* gene segment within the *Tnp* gene provides a convenient assay for studying *Tnp* gene expression. In these experiments the segment of the *Tnp* gene between the two *BclI* sites of Tn3 carrying the R49 mutation in the repressor gene was replaced (in the orientation shown for the pMC1103 plasmid, Fig. 2) by a *lac* gene-derived sequence (endonuclease-generated fragment 903 *lac*, ref. 11) that lacks a promoter but contains a translational start signal for the *lacZ* structural gene, which encodes the enzyme β -galactosidase. Expression of *lac* by pMC1103 (Table 3, line 1) verifies the proposed direction of transcription of the *Tnp* gene and demonstrates that the transcriptional start for this gene is located to the right of the *BclI* site. When a plasmid carrying a wild-type Tn3 (that is, the pACYC999 plasmid) was placed *in trans*, a decrease in β -galactosidase to 15% the level specified by the pMC1103 plasmid was observed; there was no change in the level of *lac* gene expression when a Tn3 element carrying the R49 amber mutation was introduced *in trans*, demonstrating directly that transcription of the segment of the *Tnp* gene linked to *lacZ* in pMC1103 is controlled by the repressor substance.

Suppression of the R49 amber mutation in the Tn3 repressor gene by a bacteriophage carrying an amber suppressor (λ supF, Table 2, line 4) resulted in decreased synthesis of β -galactosidase, providing additional verification that the Tn3 repressor substance regulates expression of the *Tnp* gene. The degree of suppression of the amber mutation observed in these

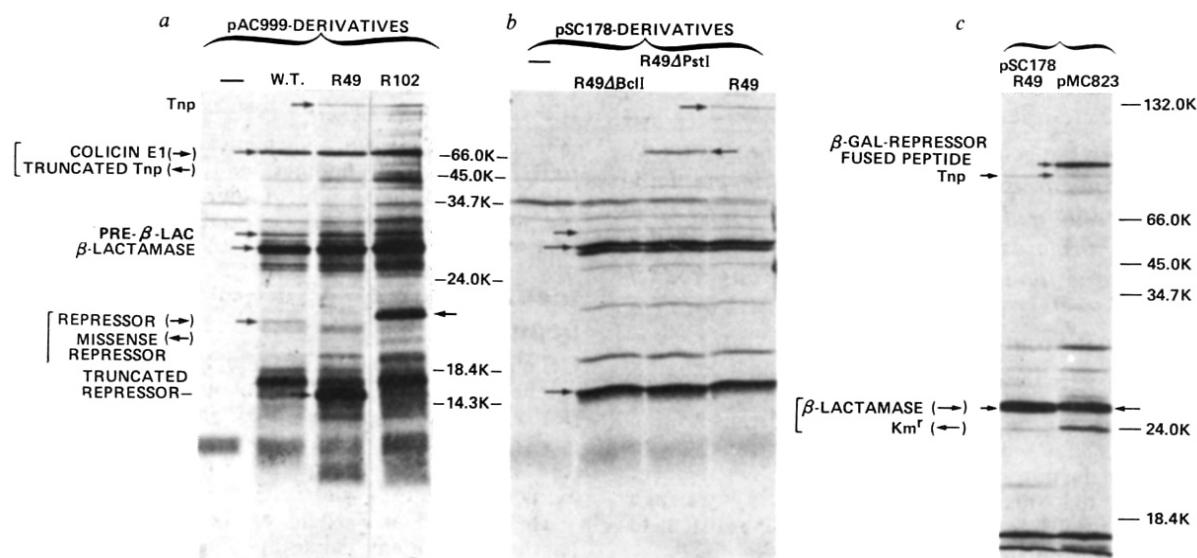


Fig. 1 Autoradiograms showing ³⁵S-labelled polypeptides synthesised by *E. coli* minicells containing plasmids carrying Tn3 or its mutant derivatives. Media used³², purification of minicells²³, and labelling²³ and electrophoresis²⁴ of peptides in polyacrylamide-SDS gels (15%, panels a and b; 10%, panel c) have been described. Molecular weight standards (between panels a and b; right-side, panel c) were lysozyme (14,300), β -lactoglobulin (18,400), trypsinogen (24,000), pepsin (34,700), ovalbumin (45,000), bovine plasma albumin (66,000), and cross-linked bovine plasma albumin (panel c only, 132,000). In panel a are shown the patterns of peptides from the minicell strain lacking a plasmid (—) and from minicells carrying the pACYC999 (ColE1::Tn3) plasmid or its derivatives, containing the R49 or R102 repressor mutations. Arrows in this panel (and in b where appropriate) indicate the positions of the 100K peptide encoded by Tn3 (*Tnp*), colicin E1 (→), the 28K β -lactamase protein and its 30K precursor (pre- β -lac)^{9,28} and the wild-type, nonsense truncated (R49), and missense (R102) Tn3 repressor peptides. The Tn3 deletion plasmids pMC1102 ($\Delta BclI$) and pMC1104 ($\Delta PstI$) (panel b) are derivatives of the pMC1097 plasmid, which is a pSC178 (pSC101::Tn3) derivative that carries the R49 amber mutation in the Tn3 repressor gene. pMC1102 and pMC1104 were constructed from pMC1097 DNA by removing internal *BclI* and *PstI* endonuclease-generated fragments of Tn3 (Fig. 2) by digestion with the respective enzymes, ligation²⁹, transformation³⁰, and selection for Ap^RTc^R in the χ 1488M minicell-producing strain (obtained from Roy Curtiss III; see also ref. 7). Selection for resistance to Ap ensures that the pMC1104 plasmid has lost only the left *PstI* fragment of Tn3. An additional arrow (←) in panel b indicates the position of a 66K truncated *Tnp* peptide synthesised by the pMC1104 ($\Delta PstI$) deletion mutant. Panel c shows patterns for the pSC178-R49 (pMC1097) and pMC823 (ref. 7) plasmids. The latter is a derivative of the pSC105::Tn3 plasmid in which the *lac* genes have been fused to the Tn3 element such that the 6th amino acid of the Tn3 repressor is joined to the 21st amino acid of the *lacZ* gene product, resulting in a fused peptide. The kanamycin-resistant pSC105::Tn3 plasmid was used as a replicon to construct pMC823 (ref. 7). This plasmid was used to provide an additional selective marker, since the β -lactamase gene of Tn3 and the Tc-resistant gene of pSC101 are both lost by deletion of the *Bam*HI-generated DNA fragment containing the IRb terminus of Tn3 to yield pMC823. Arrows in panel c indicate for pMC823 the location of the β -galactosidase-repressor fused peptide, the kanamycin resistance gene product (←), and the *Tnp* peptide. The positions of the 100K *Tnp* peptide and the β -lactamase protein (→) are shown for the pSC178-R49 plasmid.

experiments (30%) is consistent with the previously reported efficiency of amber suppressors (25–65%)¹². Analogous experiments carried out in minicells (data not shown) demonstrated a 70% reduction in the amount of the 100K Tnp peptide encoded by the pMC823 plasmid (which has deleted the entire repressor gene, see above) when the pACY999 plasmid, which carries a wild-type repressor gene, was placed *in trans*.

The level of expression of the Tnp-lac hybrid peptide encoded by the pMC1103 plasmid was found to be affected by temperature (5,217, 2,895 and 1,766 units of β -galactosidase at 30, 37 and 42 °C, respectively). These observations are consistent with the previously observed effects of temperature on translocation itself^{13,14}, and suggest that temperature may affect translocation frequency by a mechanism that controls the efficiency of transcription of the *Tnp* gene.

In the course of these experiments, we detected an additional transcriptional start signal on Tn3 using the pMC945 plasmid (Fig. 2) which was constructed by insertion of the 903 *lac* sequence into the single *Bam*HI site of the transposon; the orientation of the inserted fragment (as determined by gel analysis, unpublished data) suggests that *lac* gene expression encoded by the pMC945 plasmid results from the presence of a transcriptional start signal located towards or past the carboxy-terminal end of the repressor gene and initiating leftward transcription through the repressor gene. We have not characterised this putative promoter further, and do not know whether it has a biologically significant role. No additional Tn3 peptide assignable to this region has been identified in minicells. The promoter detected by insertion of the same 903 *lac* fusion fragment into the *Bam*HI site in the opposite orientation (that is, the pMC959 plasmid, Fig. 2) accomplishes expression of the Tn3 repressor gene and is repressible by a wild-type Tn3 *in trans* as expected (see ref. 7).

Localisation of the regulatory signals for the *Tnp* gene and determination of translational reading frame

The location and direction of reading of the repressor-controlled gene encoding the Tnp peptide suggested that its promoter is located near the promoter for the repressor gene, which is transcribed in the opposite direction. To investigate the genetic control of the *Tnp* gene and to identify more precisely where transcription and translation of this gene begin, we used

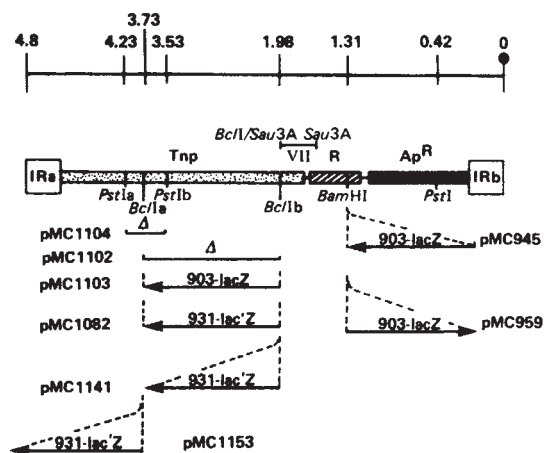


Fig. 2 Schematic map showing structure of *lac* insertions into Tn3. *Tnp* is the transposase gene, *R* the repressor gene, and *Ap* the β -lactamase gene. *IRa* and *IRb* are the inverted repeat termini of Tn3. The kilobase coordinates of restriction endonuclease cleavage sites discussed in the text are indicated; additional *Sau*3A sites within Tn3 are not shown. Kilobase coordinates are given relative to the right termini of Tn3 at *IRa*. The position of *Sau*3A fragment VII, which contains promoters and translational start signals for genes that are encoded in divergent directions is shown; the left terminus of this fragment is also the *Bcl*I cleavage site. The segments deleted in the pMC1104 plasmid (Δ PstI) and pMC1102 plasmid (Δ BclI) are indicated. The 38-base-pair-long inverted repeat termini of Tn3 (*IRa* and *IRb*) are not drawn to scale. The underlying arrows indicate the direction of insertion of 7-kilobase *Bam*HI-generated fragments containing *lacZ* and *lacZ* genes that lack promoters¹¹. 903-*lacZ* is missing only the promoter for *lacZ* transcription while 931-*lacZ* lacks both the promoter and a translational start site. The 931-*lacZ* segment starts with the same *lac* sequence shown in Fig. 3. Insertions of *lac* gene fragments in the *Bam*HI site of Tn3 were obtained by transformation of *E. coli* strain pMC1050 with ligated mixtures of one of the *lac* fragments and *Bam*HI-cleaved pACYC999 DNA, which contains only one *Bam*HI site in Tn3 and none within the *Col*E1 segment. *lac* insertions in the *Bcl*I sites were obtained by cloning the *lac* fragments into partially *Bcl*I-cleaved pSC101::Tn3 R49 DNA (the pMC1097 plasmid) which has two *Bcl*I sites within Tn3 and none in the pSC101 segment. The *Bam*HI and *Bcl*I enzymes yield identical complementary protruding ends. DNA to be cleaved by *Bcl*I was grown in a *dam*⁻ *dcm*⁻ methylase-deficient strain since methylation of DNA synthesised in *E. coli* K12 precludes *Bcl*I cleavage by the *Bcl*I enzyme.

Table 1 Effect of repressor on synthesis of the Tnp protein

Experiment 1				
Plasmids	β -Lactamase (c.p.m.)	Normalisation ratio	Repressor (c.p.m.)	Transposase (c.p.m.)
(1) None	(102)*	—	(56)	(76)
(2) pACYC999 (<i>Col</i> E1::Tn3 R*)	12,880	1.0	383†	105†
(3) pACYC999 R49 (<i>Col</i> E1::Tn3 R49)	12,440	1.0	23,873†	804†
(4) pACYC999 R67 (<i>Col</i> E1::Tn3 R67)	14,340	0.9	—	824†
(5) pACYC999 R102 (<i>Col</i> E1::Tn3 R102)	21,892	0.6	3,760	507†
Experiment 2				
Plasmids	Truncated repressor (c.p.m.)	Normalisation ratio	Transposase (c.p.m.)	
(6) None	(381)	—	(92)	
(7) pACYC999 R49	45,185	1.0	4,623†	
(8) pMC1097 (pSC101::Tn3 R49)	15,966	2.8	1,963†	
(9) pMC1104 (pSC101::Tn3 R49 Δ Pst)	9,421	4.8	7,449(t)† (138)	
(10) pMC1102 (pSC101::Tn3 R49 Δ Bcl)	9,445	4.8	(74)	

The conditions for isolation and purification of chromosomeless minicells⁹ of *E. coli* strain χ 1488M and for labelling and analysis of protein in acrylamide-SDS gels have been described^{7,23,24}. The sections of the gels (shown in Fig. 1) containing the appropriate bands were excised, digested with H₂O₂, treated with catalase, and counted²⁵. The counts per minute of peptides of interest were internally normalised to either the β -lactamase band (expt 1) or the R49-amber nonsense repressor band (expt 2) by multiplying the observed counts by the ratio of counts in the internal control bands (normalisation ratio). t = truncated transposase peptide. R67 is another previously described amber nonsense mutation in the Tn3 repressor gene⁷.

* Parentheses indicate control c.p.m. determined for a slice of control gel removed from the same location as the band being analysed.

† Normalised counts.

Table 2 β -Galactosidase synthesis by *Tnp-lac* fusions plasmids

<i>lac</i> fusion plasmid	Plasmid <i>in trans</i>	β -Galactosidase (units)	Repression ratio
(1) pMC1103	—	5,300	
(2) pMC1103	pACYC999	1,100	
(3) pMC1103	pACYC999 R49	5,700	5.2 [pACYC999 R49/pACYC999]
(4) pMC1103	λ supF	3,000	1.8 [-/ λ supF]
(5) pMC1082	—	130	
(6) pMC934	—	1,200	
(7) pMC934	ColE1	1,000	
(8) pMC934	pACYC999	120	8.5 [ColE1/pACYC999]
(9) pMC995	—	500	
(10) pMC995	ColE1	320	
(11) pMC995	pACYC999	66	5.0 [ColE1/pACYC999]

The structure of most of the pMC series of plasmids is shown in Fig. 2. pMC995 is structurally identical to pMC1082 except that its replicon is pACYC177 (ref. 26) rather than pSC101 (see text). The difference in copy number of the two vectors presumably is responsible for the observed difference in level of β -galactosidase (lines 5 and 9). The pACYC999 plasmid, the R49 amber mutation, the pACYC140 plasmid and the introduction of the λ supF amber suppressor have been described previously^{7,26,27}. For β -galactosidase assays, strain MC1050 carrying the plasmids indicated was grown to appropriate cell density in minimal medium at 30 °C and assayed for the enzyme as described elsewhere¹².

lac gene fusions to isolate promoter-containing fragments of Tn3 that are repressible by a wild-type Tn3 *in trans*. Since the frequency of transposition and the synthesis of *Tnp* gene product are both regulated by the Tn3 repressor (refs 5–7 and above), we hypothesised that at least some of the fragments isolated in this way might contain the *Tnp* gene promoter.

These experiments utilised the pMC874 plasmid (Fig. 3), which includes a gene encoding resistance to kanamycin (Km) plus a *lac* gene fragment in which the regulatory region for the *lacZ* gene and the codons for eight amino acids at the amino-terminal end of this gene have been removed and a *Bam*HI cleavage site has been introduced. The segment of *lacZ* that remains determines sufficient primary structure to yield a functional β -galactosidase enzyme, which can be expressed when a DNA fragment carrying both transcriptional and translational start signals is introduced into the *Bam*HI site¹¹. In such a case, *lac* gene expression is placed under the control of a promoter, ribosomal binding site, and translational start codon on the inserted fragment and the resulting fusion peptide contains an amino-terminal end encoded by DNA sequences on the fragment.

A *Bam*HI endonuclease-generated fragment (931 *lac*) containing the *lac* gene segment derived from the pMC874 plasmid was introduced into the *Bcl*I_b site of Tn3 on pMC1097 as shown in Fig. 2 to yield the pMC1082 plasmid. Since the *Bam*HI and *Bcl*I endonucleases generate identical 4 base pair-long cohesive termini (that is, GATC), the 931 *lac* fragment can be readily inserted at either of the *Bcl*I sites identified within the *Tnp* gene or can be substituted for the Tn3 DNA fragment between *Bcl*I sites a and b. The resulting constructs bring synthesis of the *lac* gene product under the control of regulatory signals for *Tnp*; β -galactosidase level can then be used as a direct indicator of *Tnp* expression.

As seen in Table 2, β -galactosidase is made by the pMC1082 Tn3-*lac* fusion plasmid, indicating that translation (as well as transcription, as noted above) of the *Tnp* gene commences at a location to the right of the *Bcl*I_b site. A Lac⁺ phenotype was observed with this plasmid and also with pMC1141, which contains an insertion of the 931 *lac* fusion fragment into the *Bcl*I_b site of pMC1097 (Fig. 2). Since the reading frame of the DNA segments on both sides of the *Bam*HI/*Bcl*I_b junction must be the same to yield expression of *lac* in the 931 *lac* fusion, and since the translational reading frame of the *lacZ* gene segment in the fusion is known in relation to its *Bam*HI cleavage site (see Fig. 3) and the *Bcl*I_b site in the *Tnp* gene (Fig. 4), these results establish directly the reading frame for the *Tnp* gene. It is of some interest that *lac* expression from the pMC1082 fusion plasmid, which utilises both the transcriptional and translational signals of the *Tnp* gene, is less than 3% of the level of *lac* expression from pMC1103—in which the *Tnp* transcriptional promoter is linked to translational control signals of *lac*. This implies that relatively ineffective translation of a messenger

RNA species transcribed from an efficient *Tnp* gene promoter is responsible for the observed weak expression of the *lacZ* gene in pMC1082. This interpretation is supported by results that show a markedly increased level of the *Tnp* gene product as a result of mutation within the putative translational control region for the gene (M.J.C., J.C. and S.N.C., unpublished data).

To localise more precisely the transcriptional and translational control region for the *Tnp* gene, we used the pMC874 plasmid to clone and isolate a small fragment of Tn3 that could initiate Tn3-repressible expression of the *lac* gene segment. A plasmid that includes a wild-type Tn3 element (such as pACYC999, which is ColE1::Tn3) was cleaved into small pieces using the *Sau*3A endonuclease, which recognises a 4-base pair sequence (GATC) that is a subset of the *Bam*HI recognition sequence¹³, and which generates projecting ends identical to those produced by *Bam*HI. *Sau*3A-cleaved pACYC999 plasmid DNA was ligated to the *Bam*HI-digested pMC874 plasmid, and the composite molecules were introduced by transformation into *Escherichia coli* strain MC1050 which carries a *lac* deletion. One per cent of the transformants (transformation frequency 10⁵ per μ g DNA) showed a Lac⁺ phenotype on lactose-MacConkey plates containing Km (10 μ g ml⁻¹), suggesting that a *Sau*3A-generated DNA fragment(s) carrying transcriptional and translational start signals had been cloned in the appropriate orientation at the *Bam*HI site of pMC874.

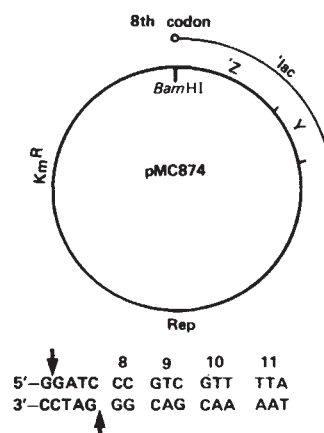


Fig. 3 Simplified map of pMC874 plasmid¹¹ indicating the position and orientation of the *lacZY* genes and the location of Km^r determinant and replication region (rep). This derivative of the pACYC177 plasmid²⁶ contains a unique *Bam*HI site, which results in cleavage before the eighth codon of the *lacZ* gene. DNA fragments that contain transcriptional signals and translational start codons yield a Lac⁺ phenotype when introduced in frame with the *lacZ* gene at the *Bam*HI cleavage site. Addition of exogenously derived amino acids to the β -galactosidase protein does not affect its activity.

Fig. 4 Partial DNA sequence of the transposase and repressor genes of Tn3. The location of the Cis₁₀ operator-constitutive mutation (which results in a C → T base change in the DNA strand corresponding to mRNA for the repressor gene)⁷ is shown in the operator-like palindromic sequence in panel *h*. Putative Pribnow box¹⁷ (PB) and Shine-Dalgarno¹⁶ (SD) sequences are shown for both the repressor and transposase genes. The translational start site for the repressor protein has previously been shown to be at the methionine codon-labelled amino acid 1 in panel *i*. The translational start site for the transposase gene, which is read in an opposite direction from that of the repressor, is assigned to the methionine codon (labelled as amino acid 1) in panel *g*. It is possible, however, that initiation of the Tnp peptide occurs at another nearby location, as discussed in the text. The positions of some endonuclease cleavage sites are indicated. *Sau*3A fragment VII of Tn3 is a 359-base pair fragment extending from the *Bcl*I/*Sau*3A site at the 46th amino acid of the transposase gene (panel *f*) to the 20th amino acid of the repressor gene (panel *i*). The DNA sequence was determined by the Maxam and Gilbert procedure²¹ by analysis of multiple fragments within the region shown, and sequences were confirmed from both strands of the duplex DNA. Details of which specific endonuclease-generated fragments were sequenced and the primary sequencing data are available from the authors on request. All fragments were labelled with [γ -³²P] ATP (~3,000 Ci mmol⁻¹, ICN) at the 5' ends using bacteriophage T4 polynucleotide kinase.

*Sau*3A fragment VII was purified from pACYC999 plasmid DNA by electrophoresis on 5% polyacrylamide gel and ligated

Fig. 5 Schematic diagram showing locations of Tn3 genes and their regulatory signals. As shown, a 21.3K repressor peptide acts to reduce transcription from the promoters for the transposase and repressor genes, which are bidirectionally expressed.

Nucleotide sequence analysis of the transcriptional and translational control region of the transposase gene

Nucleotide sequence analysis was carried out for the Tn3 DNA segment encoding the NH₂-terminal portion of the *Tnp* gene product and the regulatory region for this gene (Fig. 4). As indicated above, the 359 base-pair long *Sau*3A fragment VII of the pACYC999 plasmid contains the transcriptional and translational control region, plus the NH₂-terminal sequences for both the repressor and transposase peptides; together these genes appear to constitute a bidirectional genetic unit that regulates transposition of Tn3. Amino acids for the Tnp peptide are assigned from the ATG (AUG) codon located to the left of the single *Hae*III site within the intergenic region; no termination codon in the same frame as this ATG was seen throughout the DNA segment we have sequenced (585 base pairs). The presence on the coding strand of a GGAGG putative Shine-Dalgarno ribosomal binding sequence¹⁶ preceding the ATG codon, and a possible Pribnow box¹⁷ for binding of RNA polymerase¹⁸ several nucleotides further towards the 5' end of the coding strand, is consistent with this assignment. However, another possibility is that initiation of the Tnp peptide occurs at the GTG (GUG) codon that is 33 nucleotides nearer to the 5' end of the coding strand. This codon, which is known to be capable of initiation of translation¹⁹, is also in the correct reading frame as ascertained by our DNA sequence analysis.

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The occurrence, mid-way between the transcriptional start signals for the repressor and *Tnp* gene, of the palindromic sequence proposed by us to be the operator structure²⁰ for the repressor gene of Tn3, raises the possibility that this single operator may control both components of the bidirectional Tn3 transposition unit. Control of two promoters by a single operator region located between divergent genes has been reported previously for the bacteriophage *λ* *cI*-*cro* operon²¹.

Overall regulation of the Tn3 transposition unit

The present results, and those reported earlier⁷, provide a structural and functional basis for regulation of the transposition frequency of Tn3 and also explain the observation that structural integrity of the region to the left of the *Bam*HI site of Tn3 is required for transposition to occur^{2,3,8}. The self-regulated repressor gene is shown here to be part of a repressor-controlled divergent genetic unit (Fig. 5) that includes a gene encoding a large peptide essential for transposition of Tn3.

Transposition is thought to be a multi-step process that involves DNA nicking and ligation steps, plus replication-related functions²². The large size of the Tnp peptide may reflect an ability to carry out multiple steps in the transposition process.

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Structure and sequence of the multihaem cytochrome *c*₃

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*The molecular structure of cytochrome *c*₃ isolated from Desulfovibrio desulfuricans has been solved on the basis of its crystallographic determination at 2.5 Å resolution and of an essentially complete sequence. The molecule consists of a single polypeptide chain wrapped around a very compact core of four non-parallel haems which present a relatively high degree of exposure to the solvent. Alignment of the amino acid sequences of cytochrome *c*₃ from several species suggests that the structure reported here is characteristic of the cytochrome *c*₃ group.*

SULPHATE reducing bacteria of the genus *Desulfovibrio* are characterised by the presence of a specific electron carrier protein which has been called cytochrome *c*₃ (ref. 1). These proteins have recently been defined as a unique class of haemo-proteins which contain four haems per molecular weight of about 13,000, are autoxidisable and bear a negative oxidoreduction potential².

With the publication of the amino acid sequences of cytochromes *c*₃ from three different species of *Desulfovibrio*^{3–5}, it became clear that in spite of large differences in their amino acid composition cytochromes *c*₃ constitute a homologous class of proteins characterised by the presence of four haem attachment

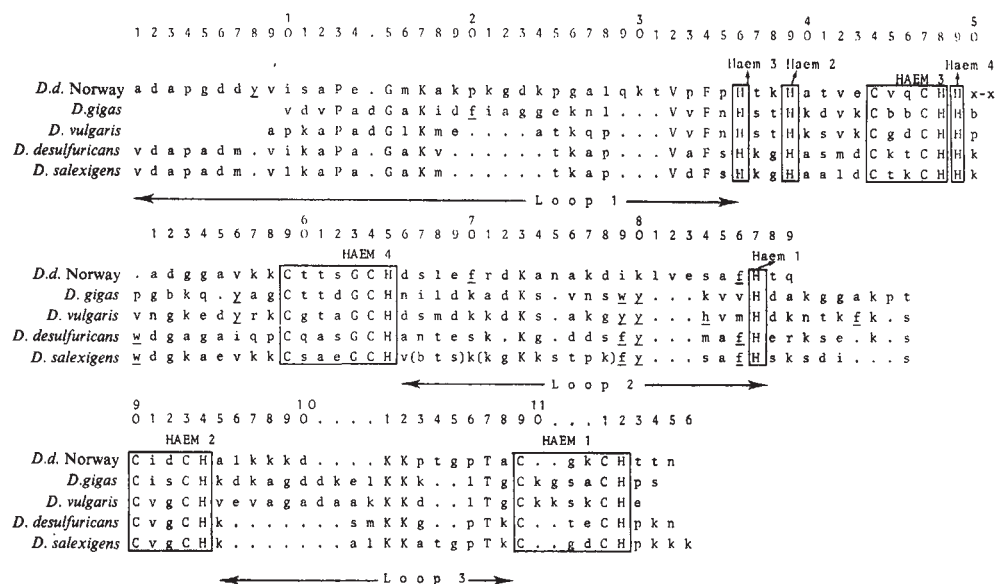


Fig. 1 Sequence homology of cytochromes c_3 . The sequences are expressed in the one letter code²⁷; *D.d. Norway* is the reference sequence; capitals indicate residues common to each sequence. Haem attachment sites are enclosed in boxes and other aromatic residues are underlined.

sites, that is, -Cys-x-x(x-x)-Cys-His-. The sequence of the cytochrome c_3 from *Desulfovibrio desulfuricans* Strain Norway (*D.d. Norway*) is almost completed and is used in the present three-dimensional study (Fig. 1).

The structural organisation of four porphyrin rings within a relatively short polypeptide chain should be quite remarkable and already extensive EPR⁶, NMR^{7,8} and Mossbauer⁹ studies have been undertaken and led to some important results: (1) the sixth axial haem ligand is a nitrogen atom of a histidine residue, therefore each iron coordination complex in cytochrome c_3 is of bis-histidinyl type; (2) close haem-haem intramolecular interactions are strongly suggested in view of short inter-iron distances (~10 Å); (3) the slow electron exchange observed between haems favours the proposal that these proteins do not act as simple electron conductors but more likely as storage devices for electrons; (4) an outline structure of *D. vulgaris* cytochrome c_3 based on sequence, EPR and NMR studies has been proposed by Dobson *et al.*⁸ and will be discussed later.

The physiological electron donor and/or acceptor for cytochrome c_3 is hydrogenase. In *Desulfovibrio* species the presence of cytochrome c_3 is a requirement for the electron transfer to occur in contrast to *Clostridium* species in which this process takes place by direct coupling between hydrogenase and low redox potential proteins such as ferredoxin, flavodoxin and rubredoxin¹⁰. Insofar as the physiological properties of the *D.d. Norway* cytochrome c_3 are known they deserve a few comments since they differ remarkably from those of cytochrome of *D. gigas* and *D. vulgaris*. In the Norway strain cytochrome c_3 has a sulphur reductase activity¹¹. Cytochrome c_3 from *D. vulgaris* also reduces sulphur but its activity is strongly inhibited by hydrogen sulphide, the product of the reaction. Another property of *D.d. Norway* cytochrome c_3 also favours a change in the function of this protein: it couples the reduction of thiosulphate from molecular hydrogen in cell-free extracts whereas *D. gigas* c_3 does not. In this latter organism this function is the property of another cytochrome absent in the Norway strain, containing eight haems per molecular weight of 26,000 and also named c_3 (ref. 12). It is noteworthy that cytochrome c_3 is not the only c -type cytochrome in *D.d. Norway* since a monohaem cytochrome $c_{553(550)}$ has recently been purified¹³.

The X-ray crystal structures of several monohaem c -type cytochromes led to the important conclusions that these molecules appear to have a similar 'cytochrome fold' and to belong to the same evolutionary group, indicating a common origin for electron transport chains in photosynthesis and respiration¹⁴⁻¹⁶. We present here the first high resolution structure of a multi-haem cytochrome in which the complete polypeptide chain

around a compact four haem arrangement could be traced throughout.

Experimental methods

Cytochrome c_3 from *D.d. Norway* was crystallised in 50% 2-methyl-2,4-pentanediol at room temperature and at pH 7.7. Rod-shaped crystals were obtained in trigonal space group $P3_1(P3_2)$ with unit cell parameters $a = b = 43.72$ Å $c = 64.45$ Å (ref. 17).

Full details of the structural analysis will be reported elsewhere but the strategy may briefly be outlined here. Three mercury derivatives were used, all prepared by soaking native crystals in appropriate solutions ($HgCl_2$, $Hg(SCN)_2$, ethylmercuriphosphate). Replicated data including Bijvoet reflections were collected on a CAD-4 Enraf-Nonius diffractometer with Ni-filtered $CuK\alpha$ radiation and an ω -scan procedure. The single site (A) of derivative $HgCl_2$ was found to be common to the three derivatives. Heavy atom parameters were refined by the F_{HLE} procedure of Dodson and Vijayan¹⁸, with empirically determined values of the real/imaginary scattering ratio¹⁹. Before, the final phase calculation combining isomorphous and anomalous measurements, accurate haem-iron coordinates were required, as the anomalous scattering from native iron atoms is far from being negligible. Native protein phases were first calculated from isomorphous data alone²⁰. The mean figure of merit for the 4,400 reflections at 2.5 Å resolution was relatively low; 0.66. At this point the electron-density map allowed a straightforward identification of the molecular boundary and of four regions of significantly higher density which obviously could be attributed to the four haem groups of the molecule. Moreover the iron atom parameters could be refined properly by a least-squares procedure using the observed native anomalous differences, in a way similar to that described for cytochrome b_5 (ref. 21). With accurate iron coordinates it was then possible to calculate a second set of 'best' phases based on both isomorphous and anomalous contributions. This led to a mean figure of merit of 0.76 falling off from 0.82 at 6 Å resolution to 0.64 at 2.5 Å resolution. The high quality of the electron-density map calculated with these improved phases can be judged from Fig. 2.

Description of the molecule

The present model is based on the polypeptide chain continuity observed on the electron density map drawn at the scale of 0.4 Å cm^{-1} and on sequence information. From this 'mini' map the high contrast between protein and solvent regions allows an easy separation between molecules. Cytochrome c_3 appears as a

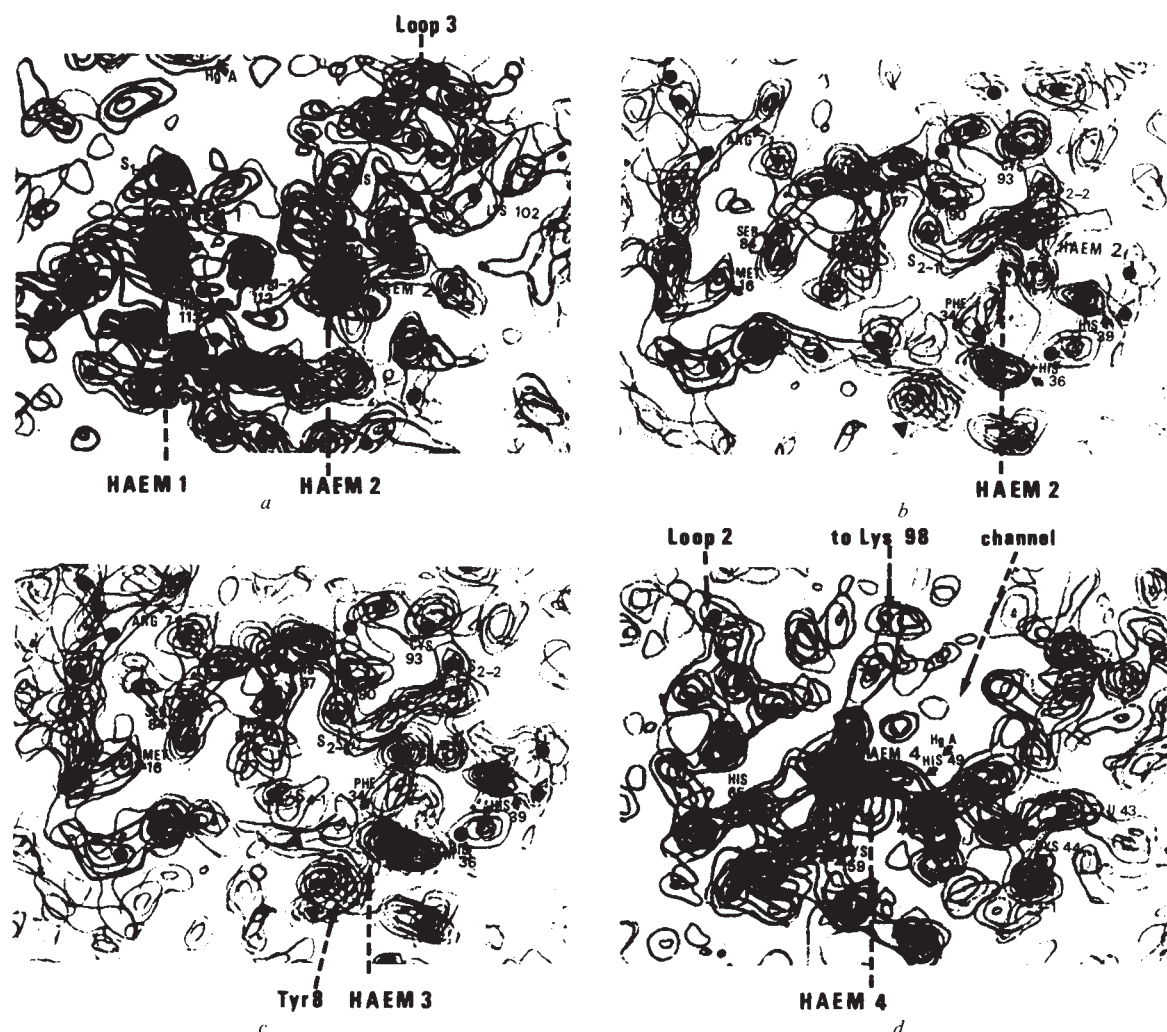


Fig. 2 The electron density map in the different haem regions. The sections are perpendicular to the three-fold axis. *a*, Haem 1 and the top of haem 2. Note the propionates of haem 2 and the loop 3; *b*, Haem 2 with His 39, Cys 90 and Cys 93; *c*, Haem 3 nearly parallel to the plane of the section. Tyr 8 is hydrogen bonded to one of its propionic acid groups; *d*, Haem 4 and the two consecutive His 48 and His 49. ●, Haem centre; ★, sulphur atom of cysteine residue; ●, α -carbon; ►, carboxylate of propionic acid.

bean-shaped molecule of overall dimensions $45 \times 28 \times 32$ Å the last figure corresponding to the three-fold axis direction. The course of the main chain could be traced entirely and the orientation of the four porphyrin rings as well as the haem binding groups are clearly visible.

The folding of the α -carbon backbone is shown diagrammatically in Figs. 3 and 4 together with the haem arrangement. Due to lack of overlap information a very small part of the sequence after His 49 has not yet been identified. Indeed the electron-density map clearly indicates the presence of one or two residues between His 49 and the next Ala and we decided to include one amino acid presently not identified and labelled X-X in position 50. A definite answer must await the final sequence data and the optimum fitting of the molecule in the Richards box. Many side chains are already identified on the mini map (Fig. 2), and are in excellent agreement with the sequence. In brief the chain consists (Figs 3 and 4) of three external loops and interconnecting haem segments, and adopts a right-handed helical conformation around residues 86–100. This helical segment runs roughly parallel to the porphyrin plane of haem 1 and contains two histidines, at residues 87 and 94, respectively linked to haems 1 and 2. At the junction of loops 2 and 3 the molecule is bent into a V-form giving rise to a deep depression. Both *N* and *C* terminals are clearly visible and lie on the surface of the molecule. Most of the hydrophilic amino acids

are located on these external loops. Among the 17 lysines and the 10 aspartic acids of the molecule, 13 and 8 respectively are found on these loops. For instance loop 3 contains the remarkable segment Lys 97-Lys-Asp-Lys-Lys 102.

Haem core

The most remarkable feature of the c_3 structure is the compact organisation of the four haems within the molecule. These lie near the surface of the protein, haems 2 and 4 sharing the same long groove dividing the molecule in two halves with one haem moiety in each. The short iron-to-iron distances which range from 10.9 Å (Fe2-Fe3) to 17.3 Å (Fe1-Fe3) reflect the close packing of the four non-parallel porphyrin rings as indicated on Figs. 3 and 4. Haems 1 and 3 are nearly parallel and roughly perpendicular to porphyrin rings 2 and 4, a spatial arrangement which does not reveal any internal symmetry.

The electron density in the neighbourhood of the four redox centres is shown on Fig. 2 where some essential features can be observed. First of all, each haem is linked, as in cytochrome *c*, to the apoprotein by two thioether bonds involving well resolved cysteines. The fifth and sixth ligands of each iron atom are provided by histidyl residues, as in cytochrome *b_5*. This is in agreement with previous NMR studies^{7,8}, ruling out Met and Lys as possible axial haem ligands. Indeed the sole methionine (Met 16) clearly visible on Fig. 2*b* and *c* is definitely not bound

Table 1 Residues close to the various haems

Haem 1	Haem 2	Haem 3	Haem 4
Val 32	Val 32, 42	Val 42, 56	x-x 50
Leu 28	Gly 105	Ala 3	Ala 85
Ala 27	Pro 33, 35	Pro 4	Gln 89
Phe 86	Pro 103, 106	His 39	Phe 86
Thr 31, 107	Phe 34	Phe 34	Lys 57, 80
Ser 84	Thr 104	Tyr 8	
	Lys 38, 97	Lys 58	

to any haem. The two consecutive histidines (His 48, 49), a highly conserved feature of all known c_3 sequences, connect directly the iron atoms 3 and 4 which correspond to the less and the most accessible haems respectively. It is to be noted that each haem group is peculiar in terms of environment and solvent exposure. Residues in the vicinity of the different redox centres are indicated in Table 1. The propionic groups located on the electron density map reflect also the various haem exposures. For haems 2 and 4, which lie in the groove largely open to the external medium, the propionates extend to the solvent whereas for haem 3 they point to the interior of the molecule: one forms a hydrogen bond with the well resolved Tyr 8 (Fig. 2c) and the second is also probably hydrogen bonded to Lys 58. Haem 1 seems to present an intermediary situation as it is partly covered by loop 1 and the C-terminal region. One of its propionic acids points towards loop 1 and is probably hydrogen bonded whereas the second appears free in the solvent. At this point some interesting intermolecular contacts are observed. One of the propionic groups of haem 4 appears to be hydrogen bonded to Lys 98 of a neighbouring molecule. This haem also faces a channel of about 7 Å diameter running throughout the crystal and providing easy access to His 49, the common binding site (A) for the three mercury derivatives (Fig. 2d).

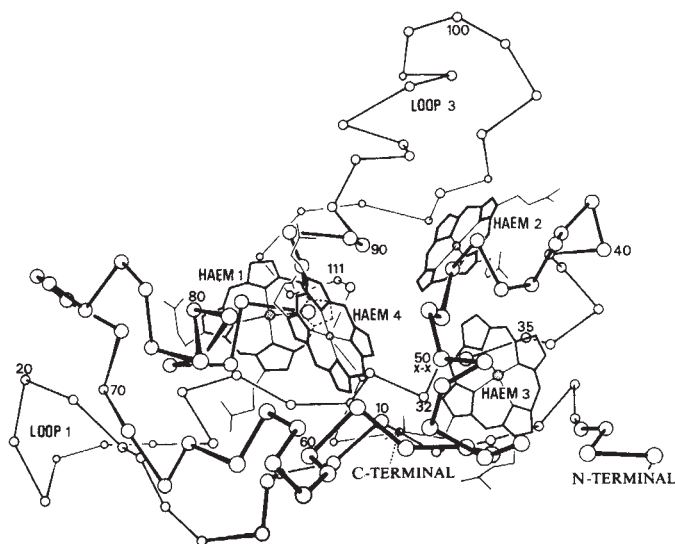


Fig. 3 A drawing of the main polypeptide chain and of the haem arrangement in cytochrome c_3 . Haem numbering is arbitrary: when looking along the three-fold axis one goes from haem 1 to haem 4 which are lying respectively near the bottom and the top of the molecule. The view is from the three-fold axis which is perpendicular to the plane of the paper. The α -carbon positions were derived from the mini map and are represented by circles whose sizes indicate their relative depth. Coordinates of haem atoms and haem ligands were measured from a Kendrew wire model built in a Richards box. Inter-iron distances are: Fe1-Fe2 = 12.7 Å; Fe1-Fe3 = 17.3 Å; Fe1-Fe4 = 16.3 Å; Fe2-Fe3 = 10.9 Å; Fe2-Fe4 = 16.8 Å; Fe3-Fe4 = 12.8 Å.

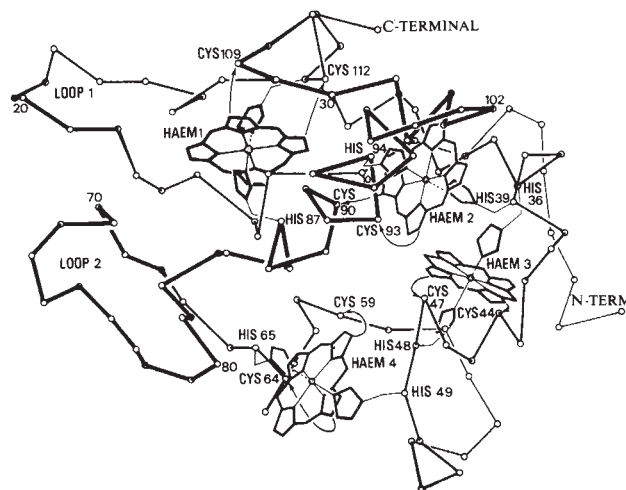


Fig. 4 The main polypeptide chain and the haem arrangement, the direction of view being approximately parallel to the a axis. Angles between porphyrin rings are 85° for haems 3-2, 80° for haems 3-4, 90° for haems 1-2, and 60° for haems 1-4. The shortest inter-haem distances (~5 Å) involving pyrrole rings are found between haems 1 and 2 and haems 2 and 3.

A detailed description of the haem environments, which should help to elucidate the intriguing problem of the haem-haem interactions, will be deferred until the completion of the model (2 cm Å⁻¹).

Discussion

The cytochromes c_3 of various species present quite different biochemical behaviours which could imply some changes in their three-dimensional structure, so the question arises as to whether the structure of *D.d.* Norway cytochrome c_3 reflects that of the other cytochromes c_3 . Comparison of primary structure of several cytochromes c_3 shows a relatively low degree of homology: among the five available sequences only 25% of the total residues remain invariant, such a variability being uncommon in the c -type cytochrome family. A possible alignment of these sequences is proposed in Fig. 1 with the *D.d.* Norway structure as reference. This alignment is based on the haem binding groups, that is the four invariant Cys—Cys—His and the four invariant histidyl residues. It is clear that most of the changes appear in the external loops and could be accommodated without disturbing the haem core. However an important modification concerns the region between His 87 and Cys 90. Examination of the reference structure suggests that this short (two amino acids) chain in *D.d.* Norway can be replaced by a longer (six to nine amino acids) external loop, and such an insertion does not necessarily imply a change in the orientation of the proximate haem 2. At present there are no structural arguments to support a different arrangement of the haem core in the cytochromes c_3 .

More subtle differences must be responsible for differentiated reactivities of cytochromes c_3 , for instance the high variability of aromatic side chains which is not encountered in the cytochrome c family. Striking evidences are shown (Fig. 1). Tyr 8 which is hydrogen bonded to the propionic group of haem 3 is specific for *D.d.* Norway which in contrast does not contain the tyrosines and tryptophans found in the other cytochromes in loop 2 or between haems 3 and 4. Phe 34 which is located between haems 2 and 3 in the *D.d.* Norway structure (Figs. 2b and c) is invariant in the five molecules suggesting that this residue is probably important for the electron transfer mechanism. On the other hand Phe 86, between haems 1 and 4 (Fig. 2c), is only found in two cytochromes. Extra aromatic residues are underlined in Fig. 1.

Despite these local variations it is likely that the three-dimensional structure reported here is representative of the

cytochrome c_3 molecule generally. In this respect the model proposed by Dobson *et al.* for *D. vulgaris* is in conflict with the actual structure. There are at least two major differences. First, the short chain segments between haems 2 and 3 and between haems 3 and 4 do not require the parallelism between the porphyrin rings but, on the contrary, haem 3 is roughly perpendicular to haem 2 and haem 4. Second, the folding suggested previously by these authors was based on the idea that the polypeptide chain is wrapped around an inner haem region. This is not true in our model with regard to the external loops and the exposure of the haem moieties as described above. In fact it would be interesting to refine the interpretation of the NMR and EPR data on the basis of our three-dimensional model. Future work should also elucidate the dependence of the redox potential on the exposure of each haem as already established for the cytochrome c family²². It is likely that such a correlation will hold for the cytochromes c_3 . As mentioned above each haem obviously has a different exposure to solvent and consequently should present a distinct reduction potential. Indeed several midpoint potentials have been measured either by EPR (ref. 6) or by differential pulse polarography²³ for different cytochromes c_3 . The lowest value $E_0' = -340$ mV for *D.d. Norway* should be assigned to the most accessible haems in the structure, namely

haem 2 or 4. Such low potentials are not detected in the three-haem cytochrome c_7 from the sulphur-reducing anaerobic bacteria *Desulforomonas acetoxidans*, $E_0' = -200$ mV. It is noteworthy that sequence comparisons show that this cytochrome c_7 can be derived from the c_3 molecule by deletion of a peptide including the binding sites of haem 4. In order to correlate these observations it will be interesting to know the three-dimensional structure of cytochrome c_7 which is now being studied in our laboratory²⁴.

Recently the interest in a detailed study of cytochrome c_3 from *D.d. Norway* has increased as it has been demonstrated that this protein acts as a very efficient electron mediator in accepting electrons from chloroplasts for the photoproduction of hydrogen from water²⁵. In this connection the cluster of four cytochromes c described as the reaction centre at the membranes of the red photosynthetic bacteria *Chromatium vinosum*²⁶ shows an intriguing similarity with the haem core of cytochrome c_3 . It is tempting to speculate that such a close proximity between the haems (8–10 Å in *C. vinosum*, as in cytochrome c_3) is an essential arrangement for electron exchange between chloroplast membrane, cytochrome c_3 and hydrogenase.

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letters

The IR energy distribution of SS433

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SS433 is an extraordinary emission-line object showing periodically varying velocities of up to $50,000 \text{ km s}^{-1}$ (refs 1, 2). It is also a variable radio source^{3,4} and an X-ray source⁵. Infrared observations of SS433 in the 1–4 μm range have been reported elsewhere^{6–10}. The data that we present here include observations at 1–10 μm and indicate first that there are substantial infrared colour variations from night to night and second that there is a sharp long-wavelength cutoff in the IR energy distribution, which was not seen in the earlier, more limited data. The discovery of this cutoff allows the diameter and electron density of the emitting region to be estimated.

Photometric measurements were made on the nights of 29, 30 and 31 May 1979 UT, using the University of Hawaii 2.2-m

telescope on Mauna Kea. The 1.2–4.8 μm observations employed an InSb detector and a 6 arc s diaphragm while the broadband 8–13 μm observations used a gallium-doped germanium bolometer and a 6 arc s diaphragm. On each night all the observations were completed within 20 min of the mean time given in Fig. 1, except that the 10 μm observation on the 29 May was made about 2.5 h earlier than the time quoted.

Figure 1 shows that between 1.2 and 4.8 μm substantial night-to-night variations exist with a suggestion that the variations are stronger at longer wavelengths. Unfortunately, no 10 μm data are available for 31 May, but a later observation by Becklin and Telesco, made during the commissioning of the 3.0-m NASA IR Telescope Facility on 1979 June 14.4 gave a 10 μm flux density of $\log S_\nu = -26.82 \pm 0.04$ a factor of 30% less than that observed 15 days earlier. At the same time C. Lonsdale and H. M. Dyck (personal communication) measured a 2.2- μm flux density of $\log S_\nu = -26.54 \pm 0.04$, a value lower than earlier measurements. The IR night-to-night variations of both flux density and colour therefore seem to be substantially larger than those reported in any other wavelength range.

The proposal by Giles *et al.*⁹ that the IR emission from SS433 is due to reddened free-free emission from an ionised plasma provides a natural explanation for the shape of the energy

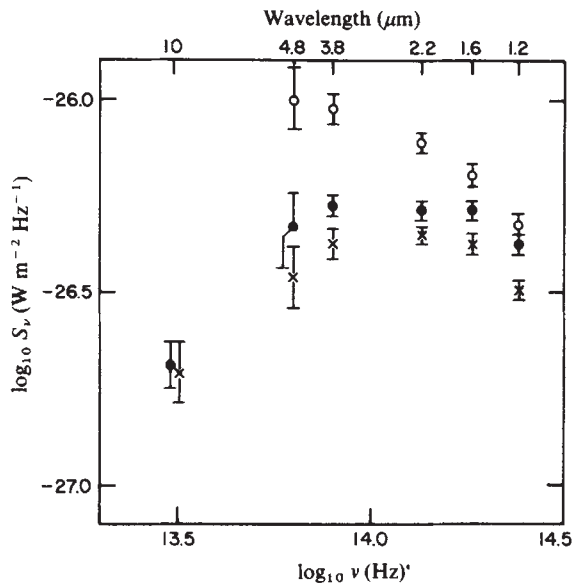


Fig. 1 The IR energy distribution of SS433 on: ●, 29.64 May 1979 UT; ×, 30.51 May 1979 UT; ○, 31.60 May 1979 UT.

distribution shown in Fig. 1, in that the decrease in flux density at wavelengths longward of $5 \mu\text{m}$ can be attributed to self-absorption. Although it is probable that the standard relationships for H II regions (see ref. 11) are inaccurate at the high column densities encountered in SS433, we may make estimates of the physical parameters of its emitting regions by assuming an electron temperature of 10^4 K and normal recombination theory. From the flux density (0.5 Jy) and the wavelength at the turnover frequency ($5 \mu\text{m}$) we can derive an angular diameter of order $2 \times 10^{-4} \text{ arcs}$ and an emission measure ($\int N_e^2 dV$) of the order of $10^{17} \text{ pc cm}^{-6}$. If a distance of 3.5 kpc is assumed¹ we estimate a physical size for the emitting region of order 1 AU , an electron density of the order of 10^{11} cm^{-3} and a photoionisation rate of 10^{48} s^{-1} , a value that is similar to that of a late O type star. The size derived here is comfortably smaller than any upper limits implied by the observed variability of the source, while the electron density is high enough to account for the weakness of the forbidden lines in the optical spectrum¹. The mass of ionised gas, assumed uniform density, is of the order of $10^{-7} M_\odot$.

If the IR radiation arises from free-free emission then a possible explanation for the fact that the variability is greater at longer wavelengths suggests itself. If an H II region is ionisation bounded its optically thin flux density is dependent on the rate of production of ionising photons, but almost independent of the geometry and electron density. At longer wavelengths, however, where self-absorption exists along at least some lines of sight the flux density is much more sensitive to changes in geometry, viewing angle and electron density. Further studies of the time variations of the IR energy distribution of SS433 may therefore help to determine the shape and the properties of its emitting region.

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Magnetic field of Jupiter and the volcanism and rotation of the Galilean satellites

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The discovery by Voyager 1 of several active volcanoes on Io^{1,2} opens up a new approach to problems such as the nature and consequences of volcanism on the celestial bodies. In the past 50 yr only Vsekhsviatky has actively considered modern volcanism on planets, in particular on Moon-like bodies^{3,4}. He proceeded from the concept of eruptive origin of comets, however, and did not suggest a physical mechanism to explain the volcanic activity he had postulated. The absence of modern volcanism on the Moon and Mars was against Vsekhsviatky's ideas and few people considered them seriously, especially in connection with the possible volcanic ejection of icy blocks. Progress was made by Peale *et al.*⁵ who predicted the existence of the volcanoes just before they were discovered, and suggested a physical mechanism for the volcanism on Io. They suggested that the tidal dissipation caused by the orbit eccentricity could produce a power $W_{\text{tid}} \approx 1.6 \times 10^{12} \text{ W}$ in Io and $8 \times 10^{10} \text{ W}$ in Europa. If the satellites are nonhomogeneous and have a molten interior, the energy release can increase several times. Peal *et al.* estimated the thickness of Io's solid crust as only 18 km . However, "how can Io's south polar region support what appear to be towering mountains? (ref. 6)". (The typical relief is $\sim 10 \text{ km}$ here²). Also, the lack of a detectable intrinsic magnetic field of Io⁶ does not favour a fully molten interior. I have previously pointed out that Io's and Europa's orbiting in the strong magnetic field of Jupiter may cause their volcanic activity⁷ and this can be confirmed by simple calculations^{8,9}. Here I shall estimate the energetics in more detail and discuss some peculiarities of the volcanism itself and consequences of the electromagnetic action mainly on the rocky satellites (the 'icy' volcanism will be considered elsewhere).

Most of the outer planets' satellites are located in the planetary magnetospheres. Io is unique: its interaction with the magnetosphere leads to radiation of $\sim 10^8 \text{ W}$ in the decametric band¹⁰. The radiation is believed to be caused by unipolar generation of the electric current due to Io's movement across the jovian magnetic field¹⁰⁻¹². The current flows from Io in Jupiter's plasmasphere along the magnetic force tubes down to the highest, weakly conducting ionospheric layers of the jovian atmosphere, just where the radiation is generated. The energy released by Io's interaction with Jupiter's field, has been predicted as, or even more than, $10^{15}-10^{16} \text{ W}$ but from estimations of the upper limit of Io's secular acceleration it seems that at present it cannot exceed 10^{14} W (see ref. 10). A more moderate value follows from the consideration that a current generated by the movement of a body across a magnetic field will hardly exceed the current $I_{\text{max}} = 2\pi BR_s/\mu_0$, whose own magnetic field is equal to the initial field¹³.

Table 1 presents the satellites' parameters: D , their distances to the centre of Jupiter; R_s , their radii; M_s , their masses; B , the magnetic field; $\Delta U = 2R_s v_{\text{rel}} B$, the generated potential difference; I_{max} ; $W_{\text{max}} = \Delta U I_{\text{max}}$ and power W_{rad} supplied by radioactive fission on the assumption of the chondritic composition ($5 \times 10^{-12} \text{ W kg}^{-1}$); the rocky cores of Ganymede and Callisto are assumed to be $\sim 1/3$ of their mass. For Callisto $W_{\text{max}} \ll W_{\text{rad}}$, for Ganymede $W_{\text{max}} \approx W_{\text{rad}}$, for Europa $W_{\text{max}} \gg W_{\text{rad}}$, but for Io and Amalthea $W_{\text{max}} \gg W_{\text{rad}}$. Note also that $W_{\text{max}} > W_{\text{tid}}$, although they have the same order of magnitude. The relation between W_{max} and W_{rad} suggest the existence of highly active volcanism on Io and, probably, on Europa.

Table 1 Magnetic data for Jupiter's satellites

Satellite	D (10^5 km)	R_s (km)	M_s (10^{23} kg)	B (G)	ΔU (kV)	$I_{\max}$ (10^6 A)	$W_{\max}$ (10^{12} W)	W_{rad} (10^{12} W)	Δt (yr)	ΔP (Myr) (at $P_0 = 10$ h)
Amalthea	1.81	130; 70	0.0002	0.26	35	9	0.31	0.0001	2.7×10^8	
Io	4.22	1820	0.891	0.020	414	18	7.45	0.45	3.6×10^{11}	0.186
Europa	6.71	1560	0.487	0.0051	159	3.9	0.62	0.24	3.4×10^{12}	0.25
Ganymede	10.7	2640	1.490	0.00125	113	1.6	0.18	0.25	4.7×10^{13}	0.68
Callisto	18.8	2420	1.074	0.00023	35	0.3	0.01	0.18	8.9×10^{14}	1.07
Amalthio	1.81	1820	0.891	0.26	500	228.0	114.00	0.45	3.4×10^9	0.08

When estimating Io's radio-wave generation, most of the energy is believed to release in the jovian ionosphere as its resistance is larger compared with Io's integral resistance¹². Apparently there has not been any estimation of the energy input in Io itself. Note, however, that all available estimations of any interaction parameters of Ioian and jovian field are ambiguous and unreliable¹⁰. The current values under consideration (restricted by the conductivity of the jovian atmosphere) greatly exceed 10^5 A (ref. 14): for instance, $I = 5 \times 10^6$ A (ref. 15), up to $I = I_{\max}$ and even sometimes considerably higher than $I_{\max}$ ($I = 2.5 \times 10^8$ A follows from $W = 10^{14}$ W). Note that the current is limited by conductivity only at $I < I_{\max}$. Also, even if the conductivity is large, I remains nearly equal to $I_{\max}$ (ref. 13). Thus, one can take $I \approx I_{\max}$ as a characteristic value, which is confirmed by the direct measurement by Voyager⁸ of the oppositely directed currents, flowing along one wing of Io-flux tube. The current was estimated as 4.8×10^6 A, hence the total current through Io's body may be as high as, or even more than, 9.6×10^6 A; thus it approaches the value of $I_{\max}$ (the currents in the opposite wings of the tube may not be equal and their strength may vary considerably with time¹⁴). The above choice is also based on the consideration that when the current flows through a gas producing an additional ionisation, and when it flows through solid dielectrics or semiconductors, heating them and ultimately causing thermal breakdown, the volt-current characteristics have a negative slope and the current stabilisation is achieved by other means such as a ballast resistance or, as in this case, by the condition $I \leq I_{\max}$. Sometimes, an opinion is expressed about the small conductance of Io^{16,17} based on the low conductivity of the outermost layers of the Moon with thickness ≤ 1 km, and then it is concluded that the current closes not through Io itself, but through its ionosphere. Clearly, however, any tenuous ionosphere should be momentarily stripped off by the Ampere forces if the magnetic field slips freely relative to nonconducting satellite, but it may exist if the current penetrates the satellite and the field is frozen somehow in its body. Voyager observations¹⁸ reveal numerous warm surface spots on Io, the temperature of which are 200–290 K and maybe even higher. These spots could be entrances for the electric current in the satellite's body through the thin outermost layer. Very crude estimations of the rocky satellite resistance can then be made using available data for the Moon¹⁹. (The conductivity σ of dirty ices from which the icy satellite crust is formed is higher compared with rocks, about 10^{-4} S, ref. 20). The main input in the resistance comes from the outer 175 km; here $\sigma = 10^{-6}$ S. Then the column with base 1 m^2 has resistance $\sim 2 \times 10^{11} \Omega$, and all the satellite body $\sim 2 \times 2 \times 10^{11} / \pi R_s^2 \Omega$. It gives for Io, 0.04 Ω ; for Europa, 0.06 Ω , which is close to values needed for $I = I_{\max}$. Thus $W_{\max}$ is the right order of magnitude for the additional energy input in the satellite body. In fact, the resistance will be somewhat lower: in the heated satellites the thickness of the low-conductivity crust is smaller and, due to the exponential dependence of conductivity on temperature, the current in the crust will be localised in some separate overheated channels (by analogy to the thermal electric breakdown of dielectrics²¹). In this way one can imagine how the aforementioned surface warm spots are created. It is in these channels that the main energy will be released and hence they will act as natural centres of volcanism. Owing to such 'high quality' input of energy, the volcanoes should appear even at $W_{\max} < W_{\text{rad}}$. The present mechanism of the volcanic activity excitation has

advantages over the tidal mechanism where the main energy is released in the centre of a satellite and at high latitudes²².

The slow non-synchronous rotation of the satellites (see below) and the maximum electrical field created between two diametrically opposite points mean that the volcanic channels have to be localised primarily in the low and middle latitudes. And indeed, all the known volcanoes and warm spots are at latitudes $\leq 30^\circ$ (refs 2, 18). This new type of volcanism indicates that a new type of convection predominates in the crust: for example, the overflowing onto the surface lavas hardens; the crust sinks due to the additional load and its lowest layer melts and then it flows up-wards through volcanoes. The present rate of Io's resurfacing due to volcanic rocky deposits may be 1 mm or more per year²³. So during 4,000 Myr all the satellite matter should make more than one convective cycle. This convection does not exclude the global convection, when because excess energy is liberated in the low latitudes the surface matter shifts to the poles (in the case of the tidal mechanism, in the opposite direction). Modern conceptions on the Earth's tectonism, suggest that the collision of the surface plates near the poles give rise to mountains. The towering mountains in the polar regions (see above) do exist.

The comparison of $W_{\max}$ with W_{rad} for galilean satellites suggests that all of them had initially large amounts of ice but the inner satellites received so much excess energy that the ice was lost.

Intensive explosions from Io's volcanoes accelerating stones up to, and more than 1 km s^{-1} imply the Plinian type of eruption and indicate that a considerable amount of volatiles exist inside Io, contrary to the ancient lunar volcanism which was volatile-poor and so developed mainly basaltic floods²⁴. To eject bombs at a velocity 0.5 km s^{-1} the volcanic activity products must contain on the average $\sim 10\%$ gases by weight²⁵. An estimated oxygen concentration in Io's plasma torus seems to be much more than the sulphur concentration²⁶, so the main gas component ejected is probably not SO_2 , which aerodynamically is not the best driving medium, but water, as in the Earth volcanoes (so it would be worth attempting to find H_2O and OH in the torus and, essentially, in volcano plumes). Practically all ejected gases escape from Io. Taking into account the present rocky deposition rate 1 mm yr^{-1} (density $\rho = 3 \text{ g cm}^{-3}$), one should obtain the ice layer ($\rho = 1 \text{ g cm}^{-3}$) lost during 4,000 Myr as thick as $\sim 1,000$ km. Thus it seems reasonable to regard the ancient Io as a Ganymede-like icy body, and its modern rocky volcanic face as a recent acquisition.

Table 1 presents data for Amalthea and 'Amalthio' (an imaginary satellite having Io's parameters but located on Amalthea's orbit). Amalthea's orbit is interesting for three reasons: (1) it is very close to the Roche limit of Jupiter, (2) it practically coincides with the orbit where the value of $W_{\max} (\gg W_{\text{rad}})$ becomes greatest, and (3) it lies well inside $4-5R_s \approx$ the radius of proto-Jupiter when it set into hydrostatic equilibrium after its rapid collapse caused by the molecular hydrogen dissociation²⁷ (here proto-Jupiter became sufficiently dense compared with the previous stage when it filled its critical Roche lobe, $R \approx 500R_s$, ref. 28). It would be natural to assume that five, not four, 'galilean' satellites existed initially, but Amalthio has been literally incinerated by Jupiter-Thunderer—by the unipolar electric currents (even now Amalthea is warmer than was expected¹⁸) and by the hypersonic and radiative ablation. It is difficult to elucidate the relative role of these factors in the

formation of the present Amalthea. It must be surely a remnant of a dense metallic core of Amalthea, strongly elongated by the ablation and bearing traces of the latter. But at some stage, matter could be dispersed by volcanic eruptions. As in the volcanism type under consideration the energy is released directly in the volcano throat, its transformation into the energy of eruptions must have a great efficiency, up to a few per cent. The energy of Amalthea's matter dispersion into space is $\sim GM_s^2/R_s = 3 \times 10^{29}$ J, so at the energy input $W_{\max} \approx 10^{14}$ W it takes a few thousand Myr to disintegrate the satellite. In reality, the convection and probably the rotation of young Jupiter were much more intensive²⁹, so that its past magnetic field was certainly far stronger than at present. $W_{\max} \propto B^2$ and the duration of transformation of Amalthea in Amalthea could be below 1,000 Myr. Some of Amalthea's fragments probably created the inner ring around Jupiter.

The idea of the five large satellites and their initial abundance of ice contradicts somewhat the commonly accepted 'micro-nebular' hypothesis of satellite condensation in the gaseous disk which surrounded hot proto-Jupiter²⁹, but it accords with my cosmogonical conception which considers the birth of the Solar System as a limiting case of a close binary star origin³⁰. The main planetary population of our system consists of Moon-like bodies²⁸. They were accreting inside proto-Jupiter filling its critical Roche lobe, in the vicinity of the first lagrangian point, when proto-Jupiter's matter was overflowing onto the Sun, and they were lost one-by-one owing to a rapid decreasing of proto-Jupiter's mass. When the outflowing was completed by cooling the vast and rarefied proto-Jupiter, the last moons (having the great ice abundance) were not able to free themselves and so remained in Jupiter's power as the galilean satellites.

The electric current flows through the satellites across the magnetic field, supplying them with an excess angular orbital momentum¹². The orbital momentum doubles in

$$\Delta L \approx M_s v_{\text{orb}} D / 2 I_{\max} B R_s D \\ = 1.3 \times 10^{-12} v_{\text{orb}} \rho_s R_s / B^2 \text{ yr} \quad (1)$$

This time is small for little Amalthea (Table 1) and its existence on this orbit, so close to the synchronous one ($D_{\text{syn}} = 1.6 \times 10^5$ km), would be stranger had it not been a much larger Amalthea in the past. (Amalthea's behaviour may be strongly modified by its own magnetic field of a ferromagnetic nature.)

The nonhomogeneity of the Ampere force $I \times B$ causes the axial rotation of a satellite to gain angular momentum. Some nonhomogeneity is created by the discrete current concentration in volcano throats. The main effect is probably caused by dipole inhomogeneity of the magnetic field: the greater force acts on the hemisphere facing Jupiter. So the satellite's axial rotation ω will be slower than its orbital rotation n . The torque created by the dipole component of the Jovian field is estimated as

$$T_B \approx I \frac{dB}{dD} R_s^3 = -3IBR_s^3/D \quad (2)$$

This torque is balanced by the torque applied by Jupiter to the tidal bulges of the satellite³¹

$$T_Q = \frac{3kGM_s^2 R_s^5}{2D^6} \sin 2\delta \quad (3)$$

Where k is the Love number (for the Moon $k = 0.02$, for Mercury $k = 0.05$ (ref. 31), we assume $k = 0.02$), δ is the lag angle of the bulges due to the internal friction in the satellite ($\delta \approx 0.005$ for the Moon). In strongly asynchronous rotation, the Earth-type planets behave as solid bodies and $\delta \approx \text{constant}$ is a good approximation. But in fully synchronous rotation ($n = \omega$) there must be $\delta = 0$, so that $\delta \rightarrow 0$ at $n - \omega \rightarrow 0$. Physically it is quite understandable: under the long-time load the creep begins to work and the matter flows, thus reaching the hydrostatic equilibrium. For fluids $\delta \propto (n - \omega)$ (ref. 32). We assume $\delta =$

$0.005(n - \omega)/(n - \omega)_0$, here $(n - \omega)_0 = 2\pi/P_0$ (a similar dependence has been accepted elsewhere³³⁻³⁵). Equating the torques (2) and (3) we find at $I = I_{\max}$, the values $n - \omega = 2\pi/\Delta P$, where ΔP is the period of the 'lost' revolution. At $P_0 = 10h$ the rotation of the galilean satellites relative to the synchronous position makes up $\sim 1-10$ arc min yr^{-1} (the equatorial velocity $\sim 10-100$ m yr^{-1}) (see Table 1). This is a rather small but detectable value, the measurement of which will allow us to specify the models of the satellites and the details of their interaction with Jupiter and its environment.

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Evidence of SO₂ on Io from UV observations

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Gaseous SO₂ was discovered on Io, the innermost galilean satellite of Jupiter, by the IRIS IR Michelson interferometer¹ on board Voyager 1 in March 1979. This SO₂ is likely to be associated with eruptions of the seven active volcanoes discovered at the same time by the Voyager cameras². The UV spectrum of the solar light reflected by the surface of Io should be modified by the transmission $\bar{S}(\lambda)$ of a SO₂ atmosphere and by the reflectivity $r(\lambda)$ of the surface. Both gaseous SO₂ and solid³ SO₂ present a deep absorption band around 290 nm. We have examined a published spectrum of Io taken in 1978 by the IUE Earth-orbiting telescope⁴, and found an absorption dip centred around 290 nm in the albedo curve. We attribute this dip to gaseous SO₂, with a possible contribution of solid SO₂. However, the absorption dip is smaller than if the gaseous SO₂ quantity measured by Voyager 1 in the IR were uniform. It can be better represented by an SO₂ frost controlled gaseous atmosphere and a temperature distribution of Io's surface compatible with observations.

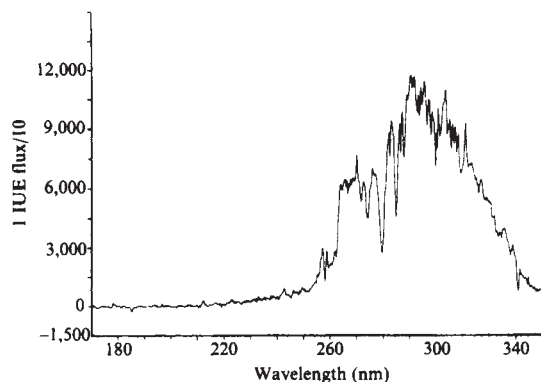


Fig. 1 The UV spectrum of Io as measured in 1978 with the orbital telescope IUE⁴.

The surface of Io, even when covered with SO₂ frost, probably does not have the same UV optical characteristics as pure SO₂ frost obtained in the laboratory, as other materials are certainly present at the surface. To conduct a quantitative analysis, we have considered successively two extreme cases: either the surface reflectivity of Io does not show the absorption dip of pure SO₂ frost (which does not exclude its presence) or it reproduces it exactly (and therefore indicates its presence).

The average surface temperature of Io on the dayside is ≈ 127 K, but some 'hot spots' exist where the temperature is probably as high as 290 K (ref. 5). This high temperature, which yields a high surface IR radiance, allowed the identification of an SO₂ absorption feature at $1,350\text{ cm}^{-1}$. The vertical column density N is estimated to be 0.2 cm atm (ref. 1) which corresponds to a surface pressure of 1.0×10^{-4} mbar. This value is close to the vapour pressure of SO₂ frost at $T = 129$ K (1.4×10^{-4} mbar). The similarity of these numbers led to suggestions that gaseous SO₂ content in Io's atmosphere is controlled by SO₂ frost. This idea is also supported by the recent identification of absorption due to SO₂ frosts in ground-based IR reflection spectra^{6,7}.

The geometric albedo of Io has been measured down to 320 nm from ground-based observations⁸ and is displayed in Fig. 2. Further in the UV, the spectrum of Io in Fig. 1 has been measured with IUE⁴, as was the spectrum of Mars. We derive the geometric albedo $G(\text{Io})$ below 320 nm from these data by using the relation:

$$\frac{G(\text{Io})}{G(\text{Mars})} = C \frac{\text{Spectrum (Io)}}{\text{Spectrum (Mars)}}$$

where C is a constant of proportionality which depends on the IUE observing conditions. The geometric albedo of Mars is obtained from measurements by OAO 2 (ref. 9), which is rather flat in this region (from 0.051 at 240 nm to 0.040 at 330 nm). The value of C was found to be 0.5 by matching the IUE albedo and the ground-based albedo at 330 nm. $G(\text{Io})$ is displayed in Fig. 2 and shows a pronounced dip, centred near 290 nm, reminiscent of the absorption coefficient K_{λ} of gaseous SO₂ also shown in Fig. 2. The vertical optical column density of 0.2 cm atm represents a vertical optical thickness $\tau_{\lambda}(\lambda) = k_{\lambda}N$ of 6×10^{-3} at 340 nm and 3.5 at 280 nm. As only the integrated albedo on the surface is available, we computed the averaged transmission $\bar{S}(\lambda)$ of various SO₂ atmosphere models, assuming that the temperature of SO₂ frost controlled the gaseous SO₂. The vapour pressure p of gaseous SO₂ over frost is a function of surface temperature T (ref. 10)

$$\log_{10} p(\mu\text{ bar}) = -1,843.04/T + 13.7164$$

and the vertical column density is $N(\text{cm atm}) = 1.96 p(\mu\text{ bar})$. Given a temperature distribution $T(\theta)$ as a function of solar zenith angle θ , the transmission $S_{\lambda}(\theta)$ of the atmosphere to reflected sunlight at 0° phase angle is $S_{\lambda}(\theta) =$

$\exp[-2k_{\lambda}N(\theta)/\cos\theta]$. If $r(\lambda)$ is the normal reflectivity of Io's surface in the absence of atmospheric absorption, the geometric albedo at 0° phase angle is:

$$G(\lambda) = r(\lambda) \int_0^{\pi/2} \cos^{n+1} \theta S_{\lambda}(\theta) d(\cos \theta) = r(\lambda) \bar{S}(\lambda)$$

which defines the equivalent transmission $\bar{S}(\lambda)$. n is an index representing the limb darkening effect. However, we present here results only for $n = 0$, as the dependence on θ is strongly dominated by the function $T(\theta)$. We have computed the equivalent transmission $\bar{S}(\lambda)$ for various temperature distributions $T(\theta)$ and have compared it with the measurement $G(\lambda)$ of Fig. 2. We take first a model for $r(\lambda)$ below 340 nm, with no SO₂ frost absorption dip, as a linear extrapolation of the trend observed above 340 nm. At 240 nm, where SO₂ absorption coefficient is small, this model (dashed line on Fig. 2) coincides with the measured $G(\lambda)$.

First we considered uniform temperature distribution. With a temperature $T = 127.2$ K giving $N = 0.2\text{ cm atm}$, we find $\bar{S}(340\text{ nm}) = 1$ (negligible absorption) and $\bar{S}(280\text{ nm}) = 2 \times 10^{-4}$ (total absorption). As $r(\lambda)$ must be < 1 , it implies that the albedo should be effectively zero at 280 nm. In fact, the non-zero albedo measured at 280 nm implies either a very low uniform value of N ($\approx 0.014\text{ cm atm}$ corresponding to $T = 117.4$ K) or a non-uniform distribution of the temperature and SO₂ column density.

We then considered models of the form

$$T(\theta) = T_s - \Delta T(1 - \cos^m \theta)$$

where T_s is the temperature at the sub-solar point, $T_s - \Delta T$ the temperature at the terminator, and m a parameter describing how fast $T(\theta)$ decreases from T_s to $T_s - \Delta T$. For each value of T_s and m , a value of ΔT can be found which satisfies the observational constraint $\bar{S}(\lambda) \approx 0.50$ at 290 nm. The value of $m = 1/4$, which provides a good empirical representation on

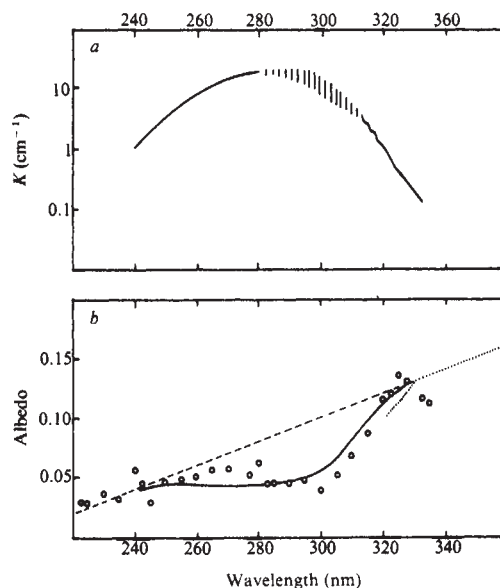


Fig. 2 *a*, The absorption coefficient K , of gaseous SO₂ as a function of wavelength¹⁰. There is a pronounced band structure between 280 and 320 nm which is represented schematically only. *b*, Geometric albedo of Io as a function of wavelength. Dotted line, ground-based measurements⁸. The linear extrapolation of ground-based measurements below 330 nm (dashed line) represents surface reflectivity variations $r(\lambda)$, if the SO₂ frost signature is ignored. $\circ$, The measurements derived from spectrum of Fig. 1, spectrum of Mars and the known albedo of Mars. The solid line is the result of a computation of the transmission of a saturated SO₂ atmosphere (averaged over the disk of Io) above SO₂ frost, containing 0.2 cm atm at the sub-solar point, and a temperature variation compatible with IR measurements.

Mercury's temperature profile, would imply small values of T , and large values of ΔT : $T_s = 123$ K and $\Delta T = 60$ K, which do not fit the IRIS data of $T_s = 127$ – 130 K, and $T = 110$ K at the evening terminator (ref. 1 and J. Pearl, personal communication). For $m = 1$, a good fit of $G(\lambda)$ is obtained with $T_s = 127$ K (corresponding to $N = 0.19$ cm atm) and $\Delta T = 34$ K and is represented in Fig. 2. This is quite reasonable, as we expect the morning terminator to be colder than the evening terminator. We, therefore, conclude that there was, at the time of IUE observations, a quantity of gaseous SO_2 comparable to the quantity detected by Voyager 1, if the temperature distribution was represented by $T = T_s - \Delta T(1 - \cos \theta)$, and if the surface reflectivity $r(\lambda)$ behaves as assumed, without any UV SO_2 frost signature.

Now we consider the other extreme case where the surface of Io has a reflectivity $r(\lambda)$ reproducing exactly the UV SO_2 frost signature. Indeed, it has been suggested³ that the decrease of geometric albedo from 330 to 320 nm (ground observations) may be due to SO_2 frost. A laboratory reflectivity spectrum of pure SO_2 frost at 130 K was recently obtained¹², showing a decrease of a factor of 28 between 325 and 275 nm. Such a decrease is clearly absent from IUE observations, which would mean in this case that only a fraction $A_f \approx 0.24$ of the planet is covered with SO_2 frost, the rest being covered with a background material with a reflectivity $\alpha_B \approx 0.064$. This is obtained

by fitting IUE observations at 275 and 325 nm with a combination of background material, pure SO_2 frost and gaseous SO_2 over this SO_2 frost. In such a case, the absorption due to gaseous SO_2 at 275 nm would be $\sim 12\%$, averaged on the whole disk.

The UV spectrum of Io shows evidence of the UV SO_2 band centred at 280 nm. To what extent this band has a contribution of SO_2 frost or is only due to gaseous SO_2 is still an open question. It could be solved by resolving the spectral structure of the gaseous SO_2 between 280 and 310 nm, absent from the pure SO_2 frost spectrum. However, our model of the temperature-controlled SO_2 atmosphere has shown that the variations of $\bar{S}(\lambda)$ are much smaller than the variations of the gaseous SO_2 absorption coefficient k_λ , requiring, therefore, accurate measurements. The monitoring of the geometric albedo spectrum, from ground observations, but more conveniently from IUE, would provide a record of changes in the atmospheric SO_2 content. This SO_2 content is a result of volcanic activity, extent of SO_2 frost deposit, temperature profile, and escape to the ionised torus discovered by Voyager 1 at the orbit of Io¹³. It would be of particular interest to observe the rise and decay times associated with a large surge of activity.

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Pioneer magnetometer observations of the Venus bow shock

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Venus has a bow shock in many respects similar to that of the Earth. The Venus bow shock was probed twice by Mariner 5, once by Mariner 10, Venera 4, and Venera 6 (refs 1–4 respectively) and repeatedly by Venera 9 and 10 (ref. 5). The Pioneer Venus orbiter is now circling Venus in a 24-h elliptical orbit which crosses the bow shock twice a day⁶. Here we report the somewhat surprising result that the Venus bow shock is not as strong as the terrestrial bow shock.

The orbit of the Pioneer Venus spacecraft is inclined at 105° to the ecliptic plane with periapsis at a latitude of 17°N and at an altitude of ~ 150 km. This orbital geometry provides shock crossings principally at solar zenith angles of greater than 60° . Figure 1 shows several recent shock crossings as recorded in the change in magnetic field strength. The shocks are ordered from top to bottom in terms of increasing θ_{BN} , the angle between the interplanetary field direction and the model⁷ shock normal. The angle, θ_{BN} , has been found to order the observed structure of the terrestrial bow shock and the waves upstream of the shock⁸. Similar control is observed here but it is not yet clear whether the control is identical to the terrestrial case.

On each panel for $\theta_{\text{BN}} \approx 50^\circ$ we have listed the field jump in the plane of the shock as determined from 1-min averages again using a shock model to determine the direction of the local shock normal. The average jump is 2.72 ± 0.16 . We have repeated this analysis for nine terrestrial shock crossings at similar solar zenith angles and angles, θ_{BN} , observed by the ISEE spacecraft during December 1977. The average field increase in the plane of the terrestrial shock was 3.37 ± 0.07 , almost 25% stronger. Further,

the overshoot in field strength so obvious in the terrestrial shock⁹ can barely be recognised in the typical Venus shock crossing.

Figure 2 shows that the weakness of the Venus shock extends over all solar zenith angles sampled to date. Here we plot the jump in the total field, not just in the plane of the shock, for 83 bow shock crossings in the range of solar zenith angles from 60° to 110° . The upper dashed line is the jump in field strength predicted by a gasdynamic simulation of the solar wind–Venus interaction.¹⁰ The Mach number of the incoming flow is 6 and the ratio of specific heats is 5/3 for this simulation. The jump in total field for our nine comparison terrestrial shocks was 3.11 ± 0.10 at a median solar zenith angle of 72° . We note that the observed field jump at the Venus bow shock does decrease with increasing solar zenith angle as in the gasdynamic simulation.

The reason for the weakness of the Venus bow shock relative to the terrestrial bow shock is not obvious. In the gas-dynamic simulation one can decrease the amplitude of the shock jump by increasing the ratio of specific heats from 5/3 to 2, but it is not evident why this ratio would be different for the two planets. We would not expect the Mach number to be significantly different at the two planets.

If the difference in shock strengths were due to some radial variation of solar wind properties we would expect that the shock at Mercury would be even weaker than the Venus shock. This is clearly not so of the two perpendicular shocks seen by Mariner 10 (refs 4, 12) one was comparable to the terrestrial shock in strength and one to the Venus shock.

We have restricted our comparison to a similar range of solar zenith angles, and in this range of angles the shock shape is the same for the two planets⁷. Thus we cannot attribute the difference in shock strengths to differing shock geometries. We must conclude that there is something basically different about the solar wind interactions with the two planets. Perhaps this difference is the existence of photo-ion pickup¹³ or charge exchange with the Venus geo-corona¹⁴. A similar conclusion is suggested by the unexpected closeness of the bow shock to Venus^{7,15}.

Fig. 1 Magnetic field strength versus time for six Pioneer Venus bow shock encounters. Sample rate is 4 samples per second. SZA is solar zenith angle, that is the angle between the Venus-Sun and Venus-spacecraft line. The angle θ_{BN} is the angle between the upstream magnetic field and a model shock normal. B_T^u is the 1-min average upstream, (interplanetary) magnetic field projected into the plane of the shock; B_T^d is the downstream magnetic field projected into the shock plane.

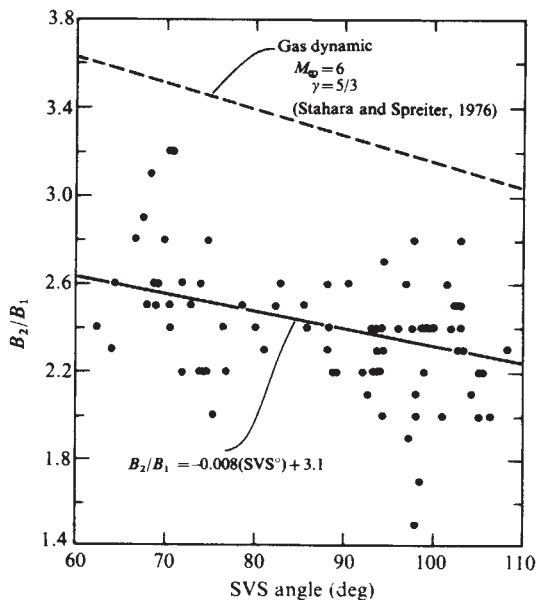
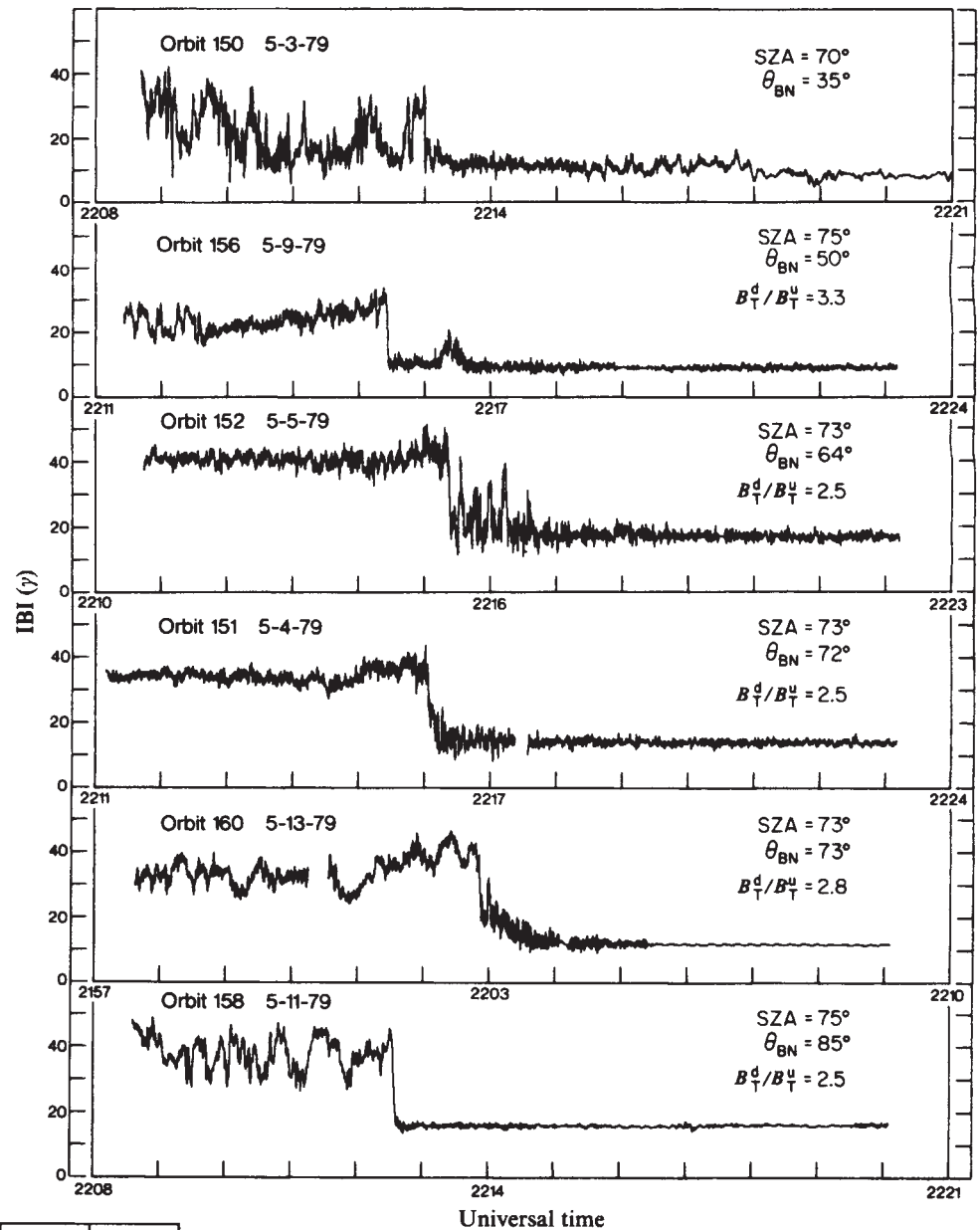


Fig. 2 The ratio of the downstream total field strength to the upstream field strength as function of the Sun-Venus-satellite or solar zenith angle. The solid line is the best least-square fit to the data, the dashed line is taken from a gas-dynamic numerical simulation.

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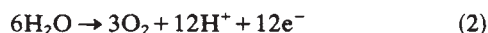
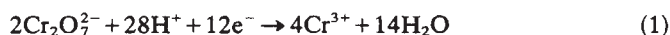
Heterogeneous photocatalytic reduction of dichromate on n-type semiconductor catalysts

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Photocatalytic and photoelectrosynthetic processes¹ are interesting from the point of view of solar energy utilisation. These processes can be developed using semiconductor electrochemistry¹⁻³. The fixing of carbon dioxide on semiconductor powders¹⁰ and amino acids synthesis on platinised TiO₂ (ref. 11) are good examples. Here we report the photocatalytic reduction of dichromate to Cr(III) on n-type semiconducting powders. This is important as dichromate is a harmful substance, and has to be treated with a reducing agent such as sodium thiosulphate to give Cr(III) in the first step of the waste treatment.

The basis of the photocatalytic reduction of dichromate is that the reaction is composed of the following two electrochemical processes;



Overall:



$$\Delta G_{298}^0 = -115.8 \text{ kJ}$$

Although the overall reaction is thermodynamically feasible, dichromate is stable in aqueous acidic solutions. As the electrochemical oxidation of water usually requires a high overpotential, the difficulty of water oxidation seems to be responsible for the stability of dichromate ions in solution. As n-type semiconductors such as TiO₂, WO₃, α -Fe₂O₃ and SrTiO₃ can photosensitise the oxidation of water^{12,13}, these must be efficient photocatalysts for the reduction of dichromate ions.

It was found that the rate of the photocatalytic reduction follows equation (4);

$$-dC/dt = k\sqrt{C} \quad (4)$$

Table 1 shows the results obtained for different types of photocatalysts. The concentration of dichromate was determined by colorimetric analysis using diphenylcarbazide as a colour former. Table 1 shows that WO₃ was the most active. The quantum yield for the photocatalytic reduction on WO₃ at $\lambda = 400 \text{ nm}$ was 0.02, as determined by ferrioxalate actinometry.

Figure 1 shows an absorption spectrum of a dichromate solution and photocurrent spectra of WO₃ and TiO₂ electrodes

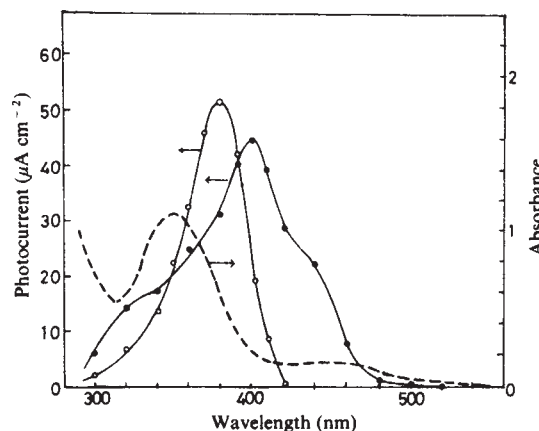


Fig. 1 Absorption spectrum of $5 \times 10^{-4} \text{ M K}_2\text{Cr}_2\text{O}_7$ in $0.5 \text{ M H}_2\text{SO}_4$ and photocurrent spectra of WO₃ and TiO₂ electrodes at 0.8 V versus SCE in $0.5 \text{ M H}_2\text{SO}_4$. ●, WO₃; ○, TiO₂. These spectra are uncorrected for intensity variation of the lamp-monochromator output, with the drop off at shorter wavelengths attributable to the lamp. The WO₃ electrode was prepared by thermal decomposition of WCl₆ (ref. 14) and the TiO₂ electrode by thermal oxidation of a titanium plate¹⁵.

in $0.5 \text{ M H}_2\text{SO}_4$. The absorption spectrum of the dichromate solution matches fairly well those of the semiconductor photocatalysts. Therefore, the light efficiency for photoexcitation of carriers is seriously weakened at the catalyst surface depending on the concentration of the solution and the thickness of the solution layer around the catalyst. The molar absorption coefficient of the dichromate solution at $\lambda = 350 \text{ nm}$, where the maximum absorption was observed, was determined to be $2.24 \times 10^3 \text{ M cm}^{-1}$, and that at $\lambda = 400 \text{ nm}$, where WO₃ shows the maximum photoresponse, to be $4.4 \times 10^2 \text{ M cm}^{-1}$.

The utilisation of solar energy in the photocatalytic reduction on WO₃ was investigated for dilute static dichromate solutions and for a concentrated agitated solution in the same way as described in Table 1. The results are given in Table 2. In spite of the high absorptivity of dichromate, the photocatalytic reduction was possible even for the solution conventionally used as a cleaning solution (no. 3 of Table 2) if that solution was stirred.

Figure 2 shows a steady-state current-potential curve of an illuminated WO₃ electrode in $0.5 \text{ M H}_2\text{SO}_4$ with and without dichromate ions. The polarisation curves were obtained with the illumination intensity adjusted to give the same anodic photocurrent at 1.2 V in both electrolytes with and without dichromate. In the solution without dichromate, the photosensitised oxidation of water occurred at potentials $>0.3 \text{ V}$ versus SCE, in agreement with the results already reported¹⁶. As Fig. 2 shows, a cathodic current peak appeared at about 0.55 V when the electrode was illuminated in the dichromate solution. If there was no illumination, the cathodic current due to the

Table 1 Photocatalytic reduction of dichromate on different types of semiconductors*

Catalyst†	λ_{th} ‡ (nm)	Purity (%)	Surface area (m ² g ⁻¹)	Amount of catalyst (g)	Decrease for 1 h (%)§	Rate constant, k (mol ^{1/2} dm ^{-1/2} h ^{-1/2})
TiO ₂ , rutile	410	99.9	9.5	0.2, 0.5, 0.8	19	1.4×10^{-2}
reduced rutile	410		8.6	0.2	37	2.9×10^{-2}
TiO ₂ , anatase	410	99.9	8.0	0.2, 0.5	13	9.5×10^{-3}
WO ₃	520	99.9	5.0	0.2, 0.4, 0.6	48	3.9×10^{-2}
α -Fe ₂ O ₃	620	99.99	3.4	0.3, 0.5, 1.1	9	3.5×10^{-3}
SrTiO ₃	390	99.9	0.67	0.2, 0.6	4	1.3×10^{-3}

* Dichromate solution, $5 \times 10^{-3} \text{ M K}_2\text{Cr}_2\text{O}_7$ in $0.5 \text{ M H}_2\text{SO}_4$. Glass reaction vessel, 15 mm diameter, 45 mm height. Solution volume, 5 ml. Light source, 1 kW xenon lamp. The amount given in the table was added to the solution, and light of $\sim 10 \text{ mm}$ diameter focused onto the agitated suspension under open air. The solution was agitated with a magnetic stirrer so that no appreciable amount of the catalyst remained on the bottom of the reaction vessel.

† Manufacturers: rutile, Fuji Titanium; anatase, Merk; WO₃, α -Fe₂O₃, and SrTiO₃, Mitsuwa Pure Chemicals.

‡ The threshold wavelength beyond which no photoresponse is observed¹⁰.

§ Almost the same value was obtained for the corresponding different amount of catalyst given.

|| Reduction in H₂ at 600°C for 3 h.

Table 2 Photocatalytic reduction of dichromate on WO₃ with sunlight

No.	Concentration of solution (mM) and volume used (ml)	Diameter of* vessel (mm)	Reaction mode	Amount of catalyst (g)	Solution above† the catalyst (mm)	Reaction time (h)	% of reaction
1	0.5‡ × 5	40	static	6	2–3	0.5	<99
2	5‡ × 5	80	static	10	<1	1	68
3	62.5‡ × 10	40	agitated	2		9	88

* Experiments were carried out in glass vessels of 40 mm diameter, 30 mm high, or of 80 mm diameter, 10 mm high covered with saran wrap.

† In the static mode of the reaction, the catalyst stayed on the bottom of the vessel.

‡ The solutions in nos 1 and 2 were prepared by dissolving dichromate in 0.5 M H₂SO₄ and that in no. 3 into 18 M H₂SO₄.

reduction of dichromate, which occurs at potentials <0.8 V with superposition of the oxygen reduction, was much smaller than that obtained under illumination and showed no current peak. The cathodic process in the dichromate solution was also enhanced at TiO₂ electrodes, although to a lesser extent than with the WO₃ electrode. No enhancement was observed at a single crystal SrTiO₃ electrode.

Figure 2 suggests that the expected electrochemical mechanism prevails in the photocatalytic reduction of dichromate, because the cathodic process of the dichromate reduction occurs at potentials greater than the onset potential of the oxidation of water. According to equations (1)–(3), the reduction to Cr(III) should be accompanied by the evolution of oxygen, but no appreciable evolution was observed in the experiments shown in Tables 1 and 2. However, small bubbles evolved intermittently from the surface of a WO₃ layer packed half way up a reaction vessel (15 mm diameter, 45 mm height) when the catalyst was kept still and xenon light was focused onto a zone of the packed WO₃/10^{−3} M dichromate solution interface.

Where the photocatalytic reaction is governed by an electrochemical mechanism, a high reaction rate will be expected if the catalyst gives high cathodic current at potentials where the photo-anodic reaction occurs. In the case of WO₃, the cathodic process is largely enhanced by illumination as stated above, so that the highest reaction rate must be achieved. The rate of the cathodic reaction seems to be governed by both the degree of the enhancement by illumination and electrocatalytic activity of the material itself in the dark. The order of the

catalytic activity shown in Table 1, that is, WO₃ > rutile > anatase > α-Fe₂O₃ > SrTiO₃, seems to reflect such a situation.

A cathodic process is not usually enhanced at n-type semiconductors, as electrons as the major carrier participate in this process. Therefore, the results obtained here are not popular in semiconductor electrochemistry, although a similar phenomenon was observed on photocatalytic oxidation of methanol on TiO₂ (ref. 17). To clarify the mechanism of the photocatalytic reduction of dichromate, this problem has to be clarified. These preliminary results indicate that photoadsorption of dichromate may have some role in the enhancement of the cathodic process.

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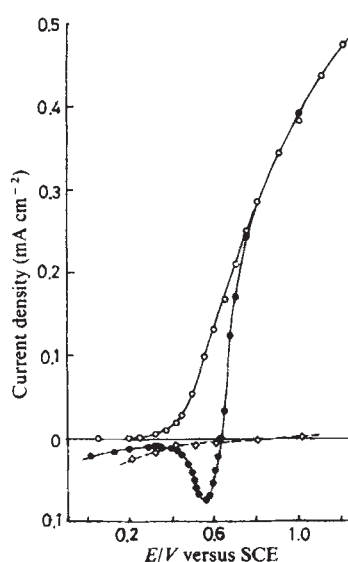


Fig. 2 Current-potential curves of a WO₃ electrode in 0.5 M H₂SO₄. O, Under illumination in oxygen-free 0.5 M H₂SO₄. ●, Under illumination in oxygen-free 0.5 M H₂SO₄ containing 5 × 10^{−3} M K₂Cr₂O₇. ◇, In the dark in aerated 0.5 M H₂SO₄ containing 5 × 10^{−3} M K₂Cr₂O₇.

Tropospheric ozone and climate

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Analysis¹ of the observed tropospheric ozone distribution has revealed that concentrations in the Northern Hemisphere were larger than in the Southern Hemisphere. This finding and the earlier identification of the necessary photochemical reaction scheme² has led Fishman and Crutzen¹ to suggest that an appreciably larger *in situ* photochemical source of tropospheric ozone may exist in the Northern Hemisphere than in the Southern Hemisphere and to speculate that anthropogenic activity might be responsible for a considerable fraction of the larger ozone concentrations in the Northern Hemisphere. In the present study, we examine the influence of tropospheric ozone on climate. Ozone is an optically active gas, which absorbs and emits terrestrial IR radiation in the 8–10-μm region and absorbs solar radiation in the UV and visible. Hence, a change in ozone concentrations will perturb the radiative energy budget of the Earth-atmosphere system which may in turn perturb the climate.

Table 1 O₃ and CO source strengths (in molecules cm⁻² s⁻¹)

	Northern Hemisphere	Southern Hemisphere	Ref.
Carbon monoxide			
Industrial CO emissions	19 × 10 ¹⁰	4 × 10 ¹⁰	7
CO from methane oxidation	4–8 × 10 ¹⁰	4–8 × 10 ¹⁰	5
Ozone			
From <i>in situ</i> production	12–32 × 10 ¹⁰	5–14 × 10 ¹⁰	5
From stratospheric injection	5–8 × 10 ¹⁰	3–4 × 10 ¹⁰	8–10

The ozone–climate problem has been studied mainly by radiative–convective models (see review in ref. 3). The recent study by Ramanathan and Dickinson⁴ revealed that a uniform percentage decrease of ozone in the troposphere has about the same net radiative cooling effect on the surface–troposphere system as the same percentage decrease of ozone in the stratosphere. This result is surprising as only about 10% of all ozone resides in the troposphere. The relatively strong sensitivity of the terrestrial radiative energy balance to a tropospheric ozone change is caused by several factors⁴, the principal one being that the integrated 9.6-μm band opacity of ozone is a function of atmospheric pressure. More specifically the line halfwidths of the individual rotational lines of the 9.6-μm band are linearly proportional to pressure, at least below 30 km. Consequently, although only ~10% of ozone is present in the troposphere, the IR opacity of tropospheric ozone is nearly the same as that of stratospheric ozone.

Our study will focus on the following aspects of the tropospheric ozone–climate problem: (1) interhemispheric differences in radiative heating of the surface–troposphere system caused by observed interhemispheric ozone distribution differences as reported by Fishman and Crutzen¹; and (2) the sensitivity of surface–troposphere radiative heating to tropospheric ozone changes. The effects are estimated as a function of latitude and season.

Several recent studies have hypothesised that ozone concentrations may have increased substantially by industrial activities in the Northern Hemisphere and will continue to grow as human activity increases^{1,5,6}. Admittedly, except in highly polluted air, it is difficult to prove, by direct observations the *in situ* photochemical production of tropospheric ozone from the oxidation of carbon monoxide and hydrocarbons in the presence of nitric oxide, because of the slow production of ozone in background air⁵. Table 1 summarises some of the studies which suggest that the amount of *in situ* photochemical production of ozone may be considerably larger than injection from the stratosphere^{5,7–10} and Table 2 presents data that show the asymmetry in both the CO and O₃ distributions between the hemispheres^{5,7,11–13}.

Note that there are several reports which either support or refute the importance of tropospheric ozone production^{1,5,6,10,14–17}. An indication that tropospheric ozone production does indeed take place in the Northern Hemisphere remote troposphere has recently been obtained from an analysis of simultaneous observation of the concentrations of CO and O₃, generally showing a positive correlation between about 2 and 8 km in the atmosphere¹¹.

Of similar importance for the production of ozone in the troposphere is the presence of hydrocarbons or carbon monoxide together with nitric oxide. The anthropogenic inputs for both CO and NO are now estimated to be comparable to the natural sources^{15–21}. As these emissions probably take place simultaneously, they are of special importance for tropospheric ozone production.

The indications support the view that the asymmetric distribution of ozone between the hemispheres may to a substantial degree be the result of human activities. However, there is no undisputable proof that tropospheric ozone concentrations have generally increased in the Northern Hemisphere over the past decades, as a sufficient long-term record of tropospheric ozone observations is not available.

We use the radiative transfer model described elsewhere⁴ which extends vertically from the surface to 54 km. Eight latitude bands are considered from the Equator to 70°, equally spaced at 10° intervals. The model computes the vertical distribution of IR and solar fluxes, using observed Northern Hemisphere distributions of temperature, humidity, clouds at three levels, CO₂, CH₄, N₂O and O₃, and surface albedo as a function of latitude and month. The radiative fluxes computed from these observed distributions are referred to as unperturbed fluxes.

The perturbation calculations discussed here are brought about by prescribing varying amounts of ozone in the troposphere; the change in stratospheric temperatures from the observed value is computed from radiative equilibrium considerations. It was found, however, that the stratospheric temperature changes had a negligible influence on the present results. The distribution of IR and solar fluxes is computed next and the differences between the fluxes in the perturbed and unperturbed troposphere are computed. The flux difference at the tropopause denotes the change in the net radiative energy input to the surface–troposphere system. It is this quantity which is considered here.

The standard profile is based on the observed distribution of ozone concentrations up to 12 km in the Northern Hemisphere as a function of month and latitude. We used three perturbations to our 'standard' profile of tropospheric ozone: (1) the ozone concentrations below 12 km were prescribed according to their distribution in the Southern Hemisphere¹; (2) ozone concentrations were arbitrarily halved at all levels from the ground to 12 km from the standard distributions; and (3) the standard ozone concentrations were arbitrarily doubled up to 12 km. The first case would simulate the present day heating difference between the two hemispheres solely because more ozone is observed in the Northern Hemisphere than in the Southern Hemisphere troposphere. The second case examines the consequences of the assumption that tropospheric ozone in the Northern Hemisphere has already doubled due to anthropogenic activities. The doubling of tropospheric ozone considered in the third case is a projection into future anthropogenic influence on this gas.

The latitudinal distributions of the annual average values of the surface–troposphere heating for the three cases are shown in Fig. 1. Considering first the interhemispheric differences, the latitudinal distribution of the difference in radiative heating rates between the Northern and Southern Hemispheres is due mainly to the latitudinal distribution of corresponding ozone concentration differences. At high latitudes, there are also significant seasonal variations in the interhemispheric ozone differences which cause corresponding seasonal variations in interhemispheric heating rate differences (not shown in Fig. 1). For example, at 50°N we calculate interhemispheric heating rates of 0.61, 0.77, 0.48 and 0.35 W m⁻² for winter, spring, summer and autumn, respectively. This seasonal variability is considerably less at low latitudes. However, more tropospheric ozone measurements in the Southern Hemisphere are needed before attaching too much importance to these seasonal variations. From Fig. 1, the hemispheric and annual mean value of the

Table 2 Observed hemispheric asymmetry of integrated CO and O₃ in the troposphere

	Asymmetry (Northern/Southern Hemisphere)	Latitudinal domain	Ref.
Carbon monoxide	1.8	90° S–90° N	7
	1.4	53° S–67° N	11
Ozone	1.3	60° S–60° N	5
	1.4	53° S–53° N	11
	1.2	90° S–90° N	12
	1.3	30° S–30° N	13

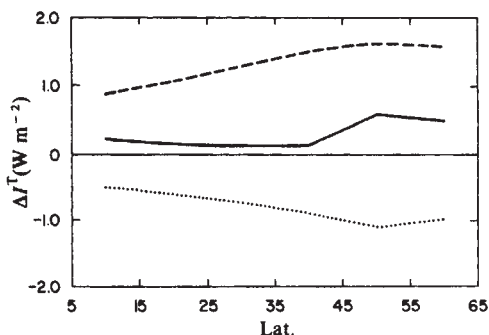


Fig. 1 Annual mean Earth-troposphere radiative heating (ΔI^T) resulting from tropospheric O_3 perturbations. Solid line, heating caused by interhemispheric O_3 differences; dotted line, heating caused by halving tropospheric O_3 ; dashed line, heating caused by doubling tropospheric O_3 .

difference in the radiative heating rates between the Northern and Southern Hemispheres is about 0.3 W m^{-2} . This difference in radiative heating can be related to a corresponding difference in surface temperature, ΔT_s , by letting

$$\Delta T_s = \frac{\Delta I^T}{dT^T/dT_s} \quad (1)$$

where the numerator is the hemispheric average of the change in the energy input to the surface-troposphere system and the denominator is the climate sensitivity parameter which has been extensively discussed during the past decade or so. Values for this parameter^{3,22} range between 0.75 and $2.5 \text{ W m}^{-2} \text{ K}^{-1}$. We will choose the estimate derived by Ramanathan²³, $dI^T/dT_s = 1.5 \text{ W m}^{-2} \text{ K}^{-1}$, which is based on Wetherald and Manabe's²⁴ general circulation model climate sensitivity analysis. Introducing the difference of 0.3 W m^{-2} in heating between the hemispheres in equation (1), we obtain $\Delta T_s \approx 0.2 \text{ K}$, that is, the Northern Hemisphere could be warmer than the Southern Hemisphere by about 0.2 K because of the larger amount of ozone in the Northern Hemisphere troposphere. Note that the Northern Hemisphere is indeed warmer than the Southern Hemisphere by about 1.0 K (ref. 25). There are, of course, other inter-hemispheric differences, such as land and ocean distribution, cloud cover, snow and ice covered areas, and water vapour concentrations which together should be more important in explaining the observed temperature difference between the hemispheres than the differences in tropospheric ozone. Nevertheless, our calculations indicate that differences in ozone may contribute appreciably to the different observed mean temperatures. Applying equation (1) to the second and third cases shown in Fig. 1, we estimate that a halving of the tropospheric ozone concentrations may cool the surface by 0.5 K and that a doubling may warm it by 0.9 K .

We feel that considerations of man's alteration of the global climate must incorporate the possibility that tropospheric ozone concentrations may increase during the coming decades. Our calculations indicate that a doubling of the tropospheric ozone content, calculated by Logan *et al.*⁶ to occur by the end of the next century, may increase surface temperatures by nearly 1 K . In a similar period of time a doubling of carbon dioxide in the atmosphere²⁶ will probably occur, which may result in a $2\text{--}3 \text{ K}$ temperature increase³. A difference between these effects is that, as a result of shorter photochemical lifetimes, the heating resulting from increasing tropospheric ozone would be more concentrated in the Northern Hemisphere, whereas the heating by carbon dioxide would be distributed more evenly between the two hemispheres. It seems possible therefore, that the photochemical production of ozone in the troposphere may enhance the potential climatic consequences of future CO_2 increases by a substantial factor, in particular in the Northern Hemisphere.

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Project Kiwi One: an acoustic cross-section of the South Pacific Ocean

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The deep ocean sound channel is used to obtain very long range (typically $>2,000 \text{ km}$) acoustic transmission¹ via totally refracted propagation paths (SOFAR propagation²). Such experiments can therefore determine the acoustic transmission properties of large areas of ocean³. Those acoustic propagation experiments can be used to locate major oceanographic changes⁴ and to identify specific water masses⁵. A 10,000-km underwater sound transmission experiment conducted between New Zealand and Peru to obtain an acoustic cross-section of the South Pacific Ocean is described here. Three distinct regions of transmission loss were found. The highest attenuation, which is attributed to Antarctic intermediate water, occurred in the central South Pacific Ocean.

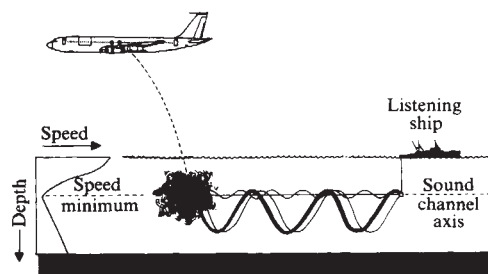


Fig. 1 Diagram of typical SOFAR propagation experiment.

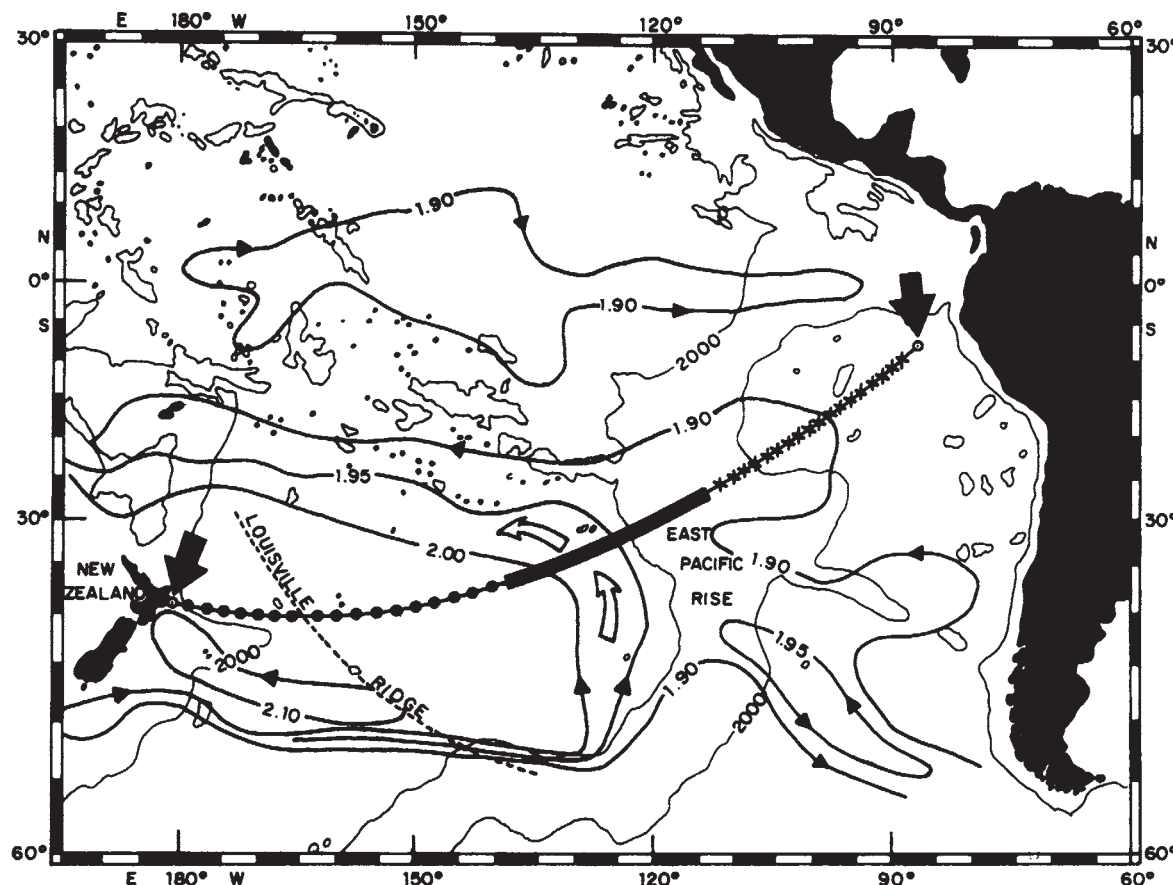


Fig. 2 Experimental track of Project Kiwi One. $\odot$ designates receiver locations. Regions: *, eastern; —, central; western. Dynamic contours (see ref. 15) indicate flow of AIW through central region (shown by arrows).

Although the South Pacific is the world's largest ocean, relatively little is known of its oceanographic and acoustic characteristics. Recent oceanographic cross-sections⁶ indicate that there is a strong influx of Antarctic intermediate water (AIW) into the South Pacific from the polar regions as part of the principal anticyclonic gyre. Results from an acoustic experiment conducted in the Tasman Sea⁷ indicate that such an influx would increase the attenuation of low frequency sound.

An ultra-long range sound transmission experiment to determine the acoustic properties and oceanographic regimes across the entire width of the South Pacific was jointly planned by US and New Zealand laboratories and designated as Project Kiwi One⁸.

In a SOFAR propagation experiment (Fig. 1) both source and receiver are located near the axis of the deep ocean sound channel, which corresponds to a minimum sound speed. A cone of sound rays follows totally refracted paths along this axis, being only attenuated by the properties of the various water masses encountered.

The track of the Kiwi One experiment (Fig. 2) extended along a great circle between the North Island of New Zealand and the coast of South America near Peru, a distance of 10,000 km. Hydrophones to receive acoustic signals were located at each end of the track. Underwater sound signals (small explosive sources which detonated at the nominal depth of the sound channel axis, 1,100 m⁹) were dropped from an aircraft at 50-km intervals along the path. Flying such a total distance non-stop was a formidable task, achieved in ~16 h by a modified Boeing 707 aircraft with inflight refuelling¹⁰. The signals received from each sound source were recorded on magnetic tape at both receiving sites and later energy analysed through 1/3 octave filter bands with centre frequencies from 32 to 250 Hz.

Typical results are shown in Fig. 3 as a function of range for centre frequencies of 32, 64, 125 and 250 Hz. The range in this

case is shown from the New Zealand receiver; similar results were obtained from the South American end. The transmission loss has been corrected for spreading loss, hence, the slope of a line fitted by a least squares analysis through the data points will give the rate at which sound is attenuated¹¹. Three distinct changes in attenuation loss occur, designated as the western, central and eastern regions, each a distance of ~3,000 km. The highest attenuation is in the central region.

A plot of the resulting attenuation coefficients as a function of frequency (Fig. 4) shows that at the higher frequencies all regions approach the predicted regional values of attenuation for the South Pacific based on pH dependent absorption¹². At

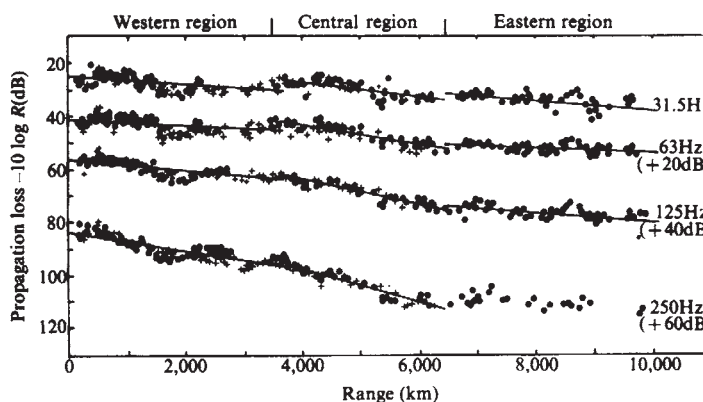


Fig. 3 Propagation loss (corrected for spreading loss) values obtained at New Zealand receiver for 1/3 octave filter bands. Levels shifted for clarity. Range is from New Zealand receiver. Fitted lines, by least square analysis, determine attenuation coefficients for each region. +, Eastbound; ●, westbound.

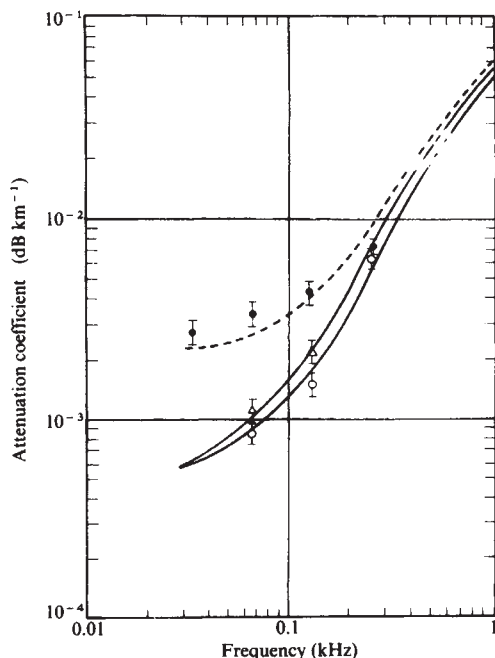


Fig. 4 Attenuation values measured in eastern region $\circ$, central region $\bullet$, western region $\triangle$ compared to attenuation obtained in Antarctic intermediate water (---), and subtropical water (—) in ref. 7. Upper solid line includes absorption component for western region, lower solid line includes absorption component for eastern region, based on ref. 12. Error bars indicate 90% confidence limits.

the lower frequencies, where it is hypothesised that the principal attenuation mechanism is scattering by oceanographic inhomogeneities¹³, a significant difference occurs.

A comparison with water mass attenuation data obtained in the South Tasman Sea, which are similarly influenced by the Antarctic circumpolar current, shows good agreement and indicates that the eastern and western regions have values of attenuation characteristic of subtropical water masses while the central region has a higher attenuation characteristic of AIW. The higher attenuation in AIW has been attributed by Kibblewhite¹⁴ to greater temperature inhomogeneities in this water mass. This influx of AIW into the central region at a depth near the sound channel axis is consistent with the Scorpio⁶ data and the dynamic flow suggested by Johnson¹⁵.

The acoustic data from a complete cross-section of the South Pacific Ocean shows three distinct regions of transmission loss, the highest occurring in the central region. The differences are due to changes in the value of low frequency attenuation. The results obtained were consistent with other attenuation measurements in Southern Hemisphere water masses and indicates that the higher attenuation in the central region is due to an influx of AIW.

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Alanine enantiomeric ratio in the combined amino acid fraction in seawater

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In deep ocean waters the concentrations of free amino acids are very close to blank levels, while surface waters contain only very low quantities^{1,2}. However, the concentration of 'combined' amino acids (which occur at all ocean depths in significant quantities) is roughly 10 times that of free amino acids^{1,2}. These combined amino acids are bound in some sort of polymeric material and are released only on hydrolysis in strong acid. The combined amino acid material apparently has a substantial D-aspartic acid content; the D/L aspartic acid ratio in the combined amino acid fraction, isolated from water from the Sargasso Sea, is ~ 0.4 at all depths². It has been suggested that combined amino acids were not actually part of the dissolved organic material in the oceans but were components of bacteria that passed through the filter used in the initial seawater-processing step^{2,3}. Although in most organisms only the L-enantiomers of the amino acids are present, bacteria contain several D-amino acids in their cell-wall proteins⁴. To verify the bacterial explanation we have carried out investigations into the enantiomeric composition of amino acids, besides aspartic acid, in the combined fraction. We measured the alanine enantiomeric ratio in seawater samples from three stations in the Pacific Ocean. Alanine was chosen because it is one of the more abundant components of the combined amino acid fraction in the oceans^{1,2}, and because D-alanine is a major component of bacterial cell-wall proteins⁴.

The combined amino acids were isolated from the seawater samples using the procedures described elsewhere^{1,2}. The D/L alanine measurements were made by gas chromatography, using the *N*-trifluoroacetyl-L-propyl peptide methyl ester method⁵. Some of the samples were sent to Dr K. Kvenvolden (US Geological Survey, Menlo Park, California) for independent analyses. The results in Fig. 1 show that the D/L alanine ratio has a value of around 0.5 in surface waters and increases to ~ 0.8 – 1.0 in deep water at all three stations. The ratios of the other amino acids measured were in the range of ~ 0.1 – 0.2 at all stations and depths. Also, the D/L ratios obtained at Scripps are compatible with those determined by Kvenvolden's laboratory.

If the combined amino acids in the oceans were simply bacterial components, then the results in Fig. 1 indicate that in deep waters, the bacterial proteins have a D/L alanine ratio close to 1.0. We feel it is unlikely, however, that bacteria would incorporate D- and L-alanine in a proportion close to 1.0, which is the chemical equilibrium ratio for the enantiomers. Also, deep water has been sampled at widely different locations and the D/L alanine ratio never exceeds 1.0. The various water samples might contain different bacterial species that would have differing D/L alanine ratios in their cell-wall proteins, and that they might have D/L alanine ratios both less and greater than 1.0. The fact that the D/L ratios do not exceed 1.0 suggests that we are observing the product of a chemical process, not a bacterial cell-wall component.

One explanation for the high D/L alanine ratio in the combined amino acids in deep oceanic waters is that racemisation has taken place. Racemisation has been observed in a variety of fossil materials and in deep-sea sediments (see ref. 6 for review). However, estimates of the racemisation rate in the ocean^{2,7} indicate that it would require hundreds of thousands of years to produce a D/L alanine ratio of ~ 1.0 ; this is much longer than the mixing times of the oceans (see ref. 8). Alanine may, however, be a component of marine humus. As this material is

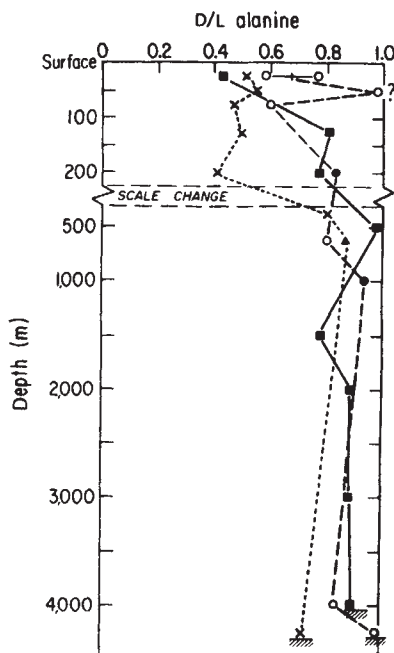


Fig. 1 The results of the D/L alanine measurements for stations: *a*, the North Pacific gyre (28°33.5' N, 155°30.7' W) (○); *b*, ~670 km south-west of San Diego (28°25.0' N, 122°31.0' W) (×); and *c*, ~60 Km off Peru (15°22.0' S, 76°02.3' W) (■). ● and ▲ are independent measurements carried out by Dr K. Kvenvolden and D. Blunt, US Geological Survey.

apparently highly unreactive and of considerable age³, racemisation might have occurred during the time that the marine humus remains in the oceans. The extent of racemisation of the various amino acids in the combined amino acid fraction seems to discount this possibility. The relative rates of the amino acid racemisation reactions⁶ indicate that aspartic acid would be more rapidly racemised than alanine, while glutamic acid should have a rate similar to alanine. This is not what we found for combined amino acids in the ocean; alanine was found to be the most highly racemised for all the amino acids.

An interesting explanation for the high D/L alanine ratios in deep water and for the increase in the D/L alanine ratio with depth in the ocean involves a dehydration reaction of serine; this reaction produces alanine that is racemic (D/L ratio of 1.0). Serine dehydration has recently been found to occur in foraminiferal tests in deep sea sediments⁹ and in fossil molluscs¹⁰. Several observations indicate that serine dehydration accounts for the high D/L alanine ratios in deep ocean waters. Our analyses indicate that serine is one of the more abundant components of the combined amino acids in surface waters, but a relatively minor component in deep waters, suggesting that this amino acid is fairly unstable and has decomposed. Whereas the serine content of the combined amino acid fraction decreases with depth, the alanine content increases; these observations are consistent with the serine dehydration pathway.

Threonine can also undergo dehydration via a pathway identical to that of serine, producing (in this case) the amino acid α -amino-*N*-butyric acid (ABA); the ABA produced by this mechanism is racemic⁹. ABA is a non-biological amino acid and is apparently not produced by any pathway other than threonine dehydration. The presence of ABA in seawater therefore would be convincing evidence that the serine and threonine dehydration reactions occur in the ocean. A peak that had a retention time similar to ABA was present on the amino acid analyser chromatograms of all of the deep water samples. The surface water samples seem to contain a trace quantity of ABA; the relative amount of ABA increased in the deep water samples relative to that in surface waters. To confirm that ABA was indeed present in deep oceanic waters, we analysed a 20-l water

sample collected from a depth of 2,800 m at a station ~670 km off the coast of southern California. We collected the fraction in which the ABA peak eluted from the amino acid analyser column. This fraction was then desalted and analysed by gas chromatography. (The procedures used for the ABA collection and the subsequent analysis are identical to those described elsewhere⁹.) The gas chromatographic analysis indicated the presence of two peaks which had retention times identical to those of the D and L enantiomers of ABA. Moreover, the gas chromatographic results indicate that the D/L ABA ratio was ~1.0, in accord with that predicted from the threonine dehydration pathway.

These various results show that the dehydration reactions of serine and threonine are quite possibly taking place in the oceans; this therefore provides some of the first experimental evidence that chemical diagenesis of dissolved organic material occurs in the sea. Alteration reactions, such as serine and threonine dehydration, may cause the organic material in the ocean to be less biologically useful, and thus they could partially explain why the bulk of the dissolved organic carbon in the oceans is of considerable age³. Nothing is known about the rate of the serine and threonine dehydration reactions in the oceans. Future investigation of the kinetics of these reactions should provide important new insights into the reactivity of the dissolved organic material in the sea.

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The surface charge of suspended particles in estuarine and coastal waters

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The colloidal stability of particles transported in suspension by rivers is controlled by surface electrical properties¹ which are conventionally regarded as undergoing rapid change during estuarine mixing¹⁻³ by interaction with polyvalent ions, especially Ca^{2+} and Mg^{2+} . Although wide differences in rates of coagulation of clay minerals as a function of salinity have been demonstrated in the laboratory^{4,5}, Gibbs⁶ and others^{7,8} have concluded that field studies provide no real evidence for the importance of such differential flocculation. Instead, changes in clay mineral distribution with distance from the source river can be accounted for by differences in the particle size spectra and the resulting sedimentation velocities. The effects of differing electrical and surface properties on coagulation rates are apparently suppressed by the formation of uniform films of metal oxides and/or organic matter around the particles⁶. We present here direct measurements of the electrokinetic charge on river and estuarine particles which confirm this hypothesis.

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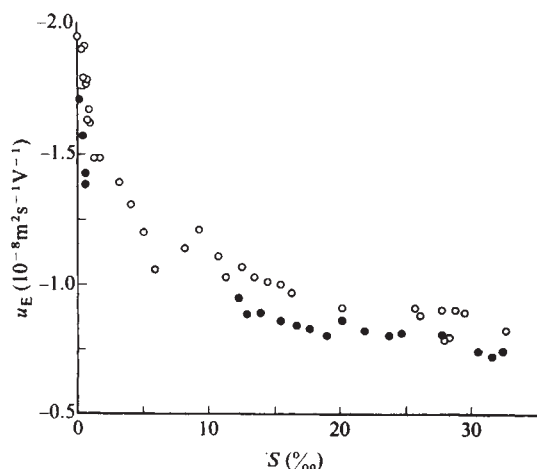


Fig. 1 Electrophoretic mobility (u_E) of particles collected in the Conwy estuary as a function of salinity (S). ●, 29 April 1979 survey; ○, 17 June 1979 survey. The electrophoretic mobility is the velocity per unit of applied electric field of particles moving at the stationary levels of the cell. Field strength was calculated from the current passed at the time of measurement and the electrolyte conductivity, determined separately. Each point plotted is the mean of determinations on at least 10 particles at each of the two stationary levels. In addition, measurements were made for all particles in each direction by reversing the field polarity. The observed standard deviation of the values was typically 14% with most in the range 6–20%. This implies a standard error in mean u_E values plotted of typically 2–4% which was confirmed by repetitive measurement of a single sample.

River and estuarine samples (20–35 per survey) have been collected from the Alde (Suffolk, 8 February and 19 March 1979), Orwell (Suffolk, 16 April 1979), Beaulieu (Hampshire, 17 May and 4 June 1979) and Conwy (North Wales, 29 April and 17 June 1979). For each sample the salinity of the water and the electrophoretic mobility of the suspended particles were measured within about a week of collection; numerous prior tests showed that storage (at 6 °C in the dark) for this period led to no significant changes in electrophoretic mobility⁹. Mobilities were determined with a Rank Mark II particle electrophoresis apparatus using a rectangular quartz cell⁹. Full details of the procedure, which utilised Ag/AgCl electrodes and a miniature measurement cell to allow determinations to be made in full strength seawater, will be published elsewhere. Samples were analysed for dissolved organic carbon (DOC) and surfactant activity in the water. The results of these analyses and the method developed for the measurement of surfactant activity will be reported separately. River waters flowing into the estuaries were analysed for their major dissolved cationic components (Na^+ , K^+ , Mg^{2+} , Ca^{2+}).

Representative plots for the relationship between electrophoretic mobility (u_E) and salinity (S) are shown in Figs 1 (Conwy) and 2 (Alde). The results for the Beaulieu are very similar to those for the Conwy. Those for the Orwell closely resemble the results given for the Alde. A composite plot giving the combined data for all seven surveys is shown in Fig. 3. The electrophoretic mobility is the appropriate parameter to be used in discussing changes in electrokinetic behaviour, because it is the only quantity which can be measured directly in this type of experiment¹⁰. Its relationship to the electrokinetic or ζ potential and the electrokinetic charge density is theoretical and depends in a complex manner on particle radius of curvature and electrolyte concentration¹¹.

Figures 1–3 clearly show that the particles are negatively charged at all salinities. The negative charge at high salinity is in agreement with previous electrophoretic measurements made directly on suspended particles in seawater^{9,12}. Figure 4 shows the results of electrophoretic measurements made on 160 individual particles in a sample of North Sea water, which

confirm the absence of positively charged surfaces. In contrast to these findings, streaming current measurements made on coarse-grained sediment particles in seawater (natural or synthetic)/distilled water mixtures indicate a change from negative to positive ζ potential at salinities which range from 1.8 to >20‰ (refs 2, 3). This discrepancy may result from differences in the sizes of particles examined by each method or in the nature of the particulate material itself. In addition, the indirect nature of the streaming current measurements may have introduced significant changes through storage effects, chemical pretreatment of the sediments, lack of equilibration with the measurement electrolytes and (in some cases) the use of synthetic media. These factors will be of particular importance if natural surface coatings are removed or altered.

It is often assumed that flocculation of riverborne particulates occurs when their charge is neutralised on mixing with high ionic strength seawater in the estuarine zone¹. The fact that all the particles examined in the present study have a substantial negative charge means that this mechanism of flocculation in estuaries is probably not important.

In the Alde and Orwell estuaries there is a gradual and almost linear decrease in negative mobility with increase in salinity. For the Conwy and Beaulieu estuaries a similar decrease occurs in the mid to upper salinity regions, but at low salinities u_E changes more dramatically. This division of the estuaries studied into two types (Figs 1 and 2) is almost certainly due to differences in river water composition. Both the Orwell and Alde rivers contain relatively high levels of dissolved cations, especially Ca^{2+} , whereas the Rivers Conwy and Beaulieu have substantially less dissolved salts. This means that in the calcareous rivers some of the natural electrokinetic charge of the particles will already be neutralised by incorporation of cations (particularly polyvalent ones) into the fixed part of the double layer, whereas for more acidic rivers, such as the Beaulieu and Conwy, this effect will not occur until the low salinity part of the estuarine zone. Support for this argument comes from laboratory experiments in which suspensions of seawater particles were mixed with high and low Ca^{2+} river waters and distilled water. Irrespective of the origin of the marine particulates, the high Ca^{2+} river water gave a u_E/S plot resembling Fig. 2, whereas low Ca^{2+} river water, and more especially distilled water, produced plots of similar shape to Fig. 1.

Apart from differences due to river water composition, the combined data in Fig. 3 show remarkably little dispersion and there is also good agreement between the results of independent surveys of the same estuary (Figs 1 and 2). Perhaps more importantly, the variations in u_E of individual samples are small (see legend to Fig. 1) indicating a high degree of surface uniformity. The results shown in Fig. 4 are typical in this respect; at

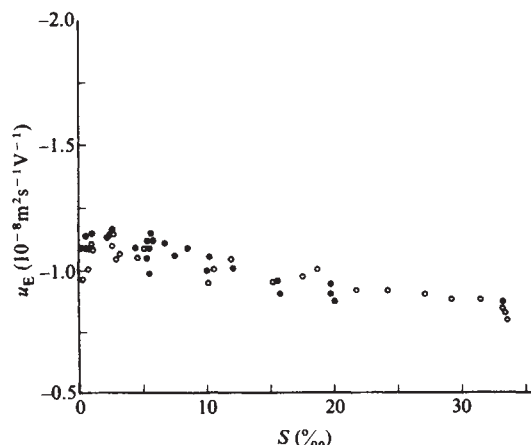


Fig. 2 Electrophoretic mobility (u_E) of particles collected in the Alde estuary as a function of salinity (S). ●, 8 February 1979 survey; ○, 19 March 1979 survey. Standard errors of mean u_E values are comparable to those of the Conwy estuary (Fig. 1).

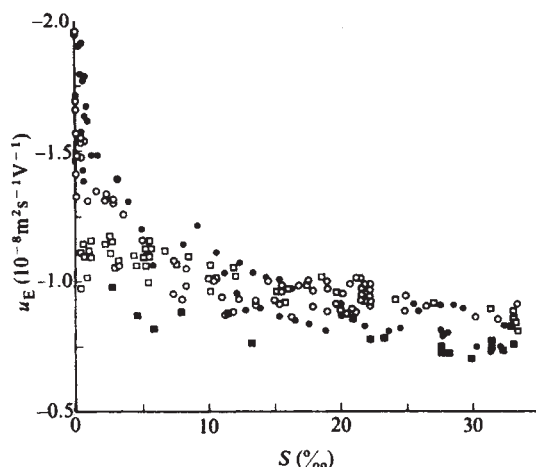


Fig. 3. Composite plot showing electrophoretic mobility (u_E) of particles as a function of salinity (S) for the four rivers studied. $\circ$, Beaulieu estuary; $\bullet$, Conwy; $\square$, Alde; $\blacksquare$, Orwell. Data for the two separate surveys of each of the first three estuaries have been combined in the one symbol.

least half of the total u_E variability (13%) in this case is attributable to experimental errors (mainly due to focusing)¹³. In view of the highly varied nature of the particle types involved (various clay minerals, aluminosilicates, quartz grains, calcite, living and dead organisms, detritus), the present results provide the first direct evidence of a dominant role of oxide and/or organic films in determining the surface properties of suspended estuarine particles. Previous work using similar methods has established that natural organic surfactants largely control the properties of surfaces introduced into seawater through the formation of cohesive films^{9,12,14,15}. Surfactant activity and DOC results for the present surveys indicate levels of adsorbable organic matter in excess of those in seawater. Accordingly, in these estuarine systems organic coatings are expected to be at least as important as in seawater. With respect to control by oxide films, the Beaulieu estuary (Fig. 3) is an area of rapid removal of dissolved iron¹⁶ and there is some evidence for uptake of the precipitated material onto particle surfaces¹⁷.

As surface active organic matter is ubiquitous in natural waters, it seems reasonable to conclude that the basic principles revealed here are sufficiently general to apply in other areas. For example, the uniformity of electrokinetic charge on suspended estuarine particulates, arising from the dominant presence of surface coatings, is able to explain the apparent lack of evidence

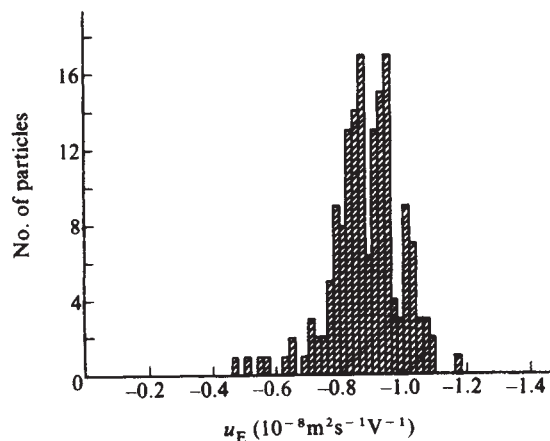


Fig. 4. Frequency histogram of electrophoretic mobility (u_E) of 160 natural suspended particles in coastal North Sea waters collected 6–7 km south-east of Great Yarmouth, Norfolk. Combined data for both stationary levels. Mean and standard deviation of u_E : $0.89 \pm 0.12 \times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$.

for differential flocculation of minerals in field studies of sediment distribution^{6–8}. Furthermore, it seems likely that surface films will exercise a major role in chemical transformations involving particulate matter. Thus, for example, uptake/release of dissolved trace metals by particles may well depend on the chemistry of the organic matter forming the film rather than that of the underlying solid matrix.

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Helium and xenon in deep-sea basalts as a measure of magmatic differentiation

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There has been much recent interest in the rare gas abundances of terrestrial materials, due mainly to the discovery of 'excess' radiogenic Ar and He trapped in deep-sea glasses^{1–4}. The abundances of these gases indicated that such materials might effectively trap the internal Earth's atmosphere, and evidence of trapped primordial gases has by now been found not only in deep-sea glasses^{5–10}, but also in xenolithic inclusions^{11–15}, deep ocean waters¹⁶, CO₂ well gas^{17–19}, volcanic gas²⁰ and diamonds²¹. The rare gas elemental abundances measured in these samples may not quantitatively reflect the deep-Earth pattern. Several elemental fractionation processes have been suggested, ranging from selective enrichment of the lighter gases in an upward-rising magma^{22,23} to post-eruptive diffusion loss which would selectively deplete the lighter gases²⁴. Each of these processes will affect concentrations of the helium and xenon isotopes in a characteristic manner, thus leaving in the glasses an isotopic fingerprint of what has occurred. New data are reported here which indicate extremely small concentrations of fissionogenic xenon in deep-sea basalts containing large concentrations of trapped excess radiogenic helium and argon. The resulting high ⁴He/¹³⁶Xe ratios are diagnostic of fractionation during the magmatic processes leading to eruption rather than of post-eruptive loss of gas (which would show the opposite effect). The results indicate that studies of volatiles in general and of the rare gases in particular in such mantle-derived samples, even in those with no subsequent alteration or contamination, may be profoundly influenced by mass-dependent pre-eruptive effects. The consequences of this fractionation for the deep-Earth K/U ratio are discussed.

Table 1 Rare gas data for tholeiitic glasses from the Vema Fracture Zone (MAR)

Sample	^4He (10^{-8} scc g $^{-1}$)	$^4\text{He}/^{40}\text{Ar}_t$	Xe (10^{-12} scc g $^{-1}$)	$^{129}\text{Xe}/^{132}\text{Xe}$	$^{131}\text{Xe}/^{132}\text{Xe}$	$^{134}\text{Xe}/^{132}\text{Xe}$	$^{136}\text{Xe}/^{132}\text{Xe}$	$^{136}\text{Xe}_t$ (10^{-15} scc g $^{-1}$)	$^4\text{He}/^{136}\text{Xe}_t$ ($\times 10^9$)
AT-72 (1)	1,500 $\pm$ 300	18 $\pm$ 2	1.4	0.980 $\pm$ 0.01	0.788 $\pm$ 0.01	0.388 $\pm$ 0.007	0.343 $\pm$ 0.006	4.8	3
(2)	—	—	13	1.00 $\pm$ 0.01	0.809 $\pm$ 0.01	0.386 $\pm$ 0.006	0.330 $\pm$ 0.005	<6	—
(4)	860	16	1.0	1.02 $\pm$ 0.01	0.768 $\pm$ 0.01	0.390 $\pm$ 0.007	0.355 $\pm$ 0.006	2.6	3.3
(7)	—	—	3.5	0.968 $\pm$ 0.01	0.796 $\pm$ 0.01	0.389 $\pm$ 0.007	0.337 $\pm$ 0.006	6.6	—
(8)	1,150 ²⁴	15 ²⁴	—	—	—	—	—	—	—
(9)	1,300 ²⁴	17 ²⁴	—	—	—	—	—	—	—
Avg.	1,200 $\pm$ 270	16 $\pm$ 1	—	—	—	—	—	4.6 $\pm$ 2	2.4 $\pm$ 2
AT-88 (1)	1,800 $\pm$ 600	9 $\pm$ 3	1.6	1.07 $\pm$ 0.03	0.807 $\pm$ 0.02	0.40 $\pm$ 0.01	0.342 $\pm$ 0.005	5.2	3.5
(2)	2,300	7.2	62	0.978 $\pm$ 0.02	0.758 $\pm$ 0.02	0.381 $\pm$ 0.008	0.328 $\pm$ 0.006	—	—
(3)	2,400 ²⁴	8.3 ²⁴	—	—	—	—	—	—	—
(4)	2,400 ²⁴	7.3 ²⁴	—	—	—	—	—	—	—
AT-92 (1)	2,400	9.3	20	0.986 $\pm$ 0.01	0.780 $\pm$ 0.01	0.374 $\pm$ 0.01	0.330 $\pm$ 0.005	—	>1
(2)	1,900	—	4.6	1.01 $\pm$ 0.02	0.778 $\pm$ 0.01	0.390 $\pm$ 0.005	0.332 $\pm$ 0.006	<8	>2
(3)	1,600 ²⁴	6.8 ²⁴	—	—	—	—	—	—	—
(4)	2,370 ²⁴	12 ²⁴	—	—	—	—	—	—	—

Absolute accuracy is $\pm 15\%$ for Ar, $\pm 20\%$ for He and Xe.

Most terrestrial ^4He is due to the radioactive decay of the U and Th families. A small contribution to terrestrial Xe is due to the spontaneous fission of U. The Th/U ratio of non-sedimentary, non-metamorphic terrestrial rocks is 3 ± 1 . The production ratio of radiogenic He ($^4\text{He}_r$) and fissiogenic Xe ($^{136}\text{Xe}_f$) is nearly constant with time and with the nearly constant Th/U ratio ($^4\text{He}_r/^{136}\text{Xe}_f = 4.2 \times 10^8 \pm 10\%$). Finally, He and Xe are the lightest and heaviest of the rare gases, respectively, and so their separation by any mass-dependent process will show the largest possible effects. Therefore a $^4\text{He}_r/^{136}\text{Xe}_f$ ratio $\sim 4.2 \times 10^8$ in a given material is evidence that no significant mass fractionation effects have disturbed the rare gas pattern, a value $< 4.2 \times 10^8$ is evidence for preferential loss of He, and a value $> 4.2 \times 10^8$ is evidence for preferential He enrichment.

Samples of glassy basalts outcropping in the Vema Fracture Zone (Mid-Atlantic Ridge), previously shown to have high $^4\text{He}_r/^{40}\text{Ar}_t$ ratios characteristic of efficient trapping of the pre-eruptive rare gases²⁴, were selected for analysis. The samples were melted and partially vaporized in an r.f.-induction furnace, and the resulting gases were purified over two successive Ti-getters at various temperatures. Helium was measured while Ar and Xe were adsorbed on charcoal; the Ar and Xe were then successively released and measured. The instrument was a Reynolds-type steel 4.5"–60" mass spectrometer. Isotopic ratios were determined by extrapolation of several spectra sweeps back to entrance time; absolute amounts were calibrated by pure gas standards of atmospheric (except for He) isotopic composition run alternatively with the samples; blank runs were interspersed. A correction for instrumental mass fractionation was made on the basis of the standard gases. The results are shown in Table 1; the Xe isotopic data are plotted in Fig. 1. The linear relationship follows from:

$$\begin{aligned}
 ^{136}\text{Xe}_t &= ^{136}\text{Xe}_f + ^{136}\text{Xe}_m + ^{136}\text{Xe}_a \\
 &= ^{136}\text{Xe}_f + a(^{132}\text{Xe}_m + ^{132}\text{Xe}_a) \\
 &= ^{136}\text{Xe}_f + a^{132}\text{Xe}_t \\
 \frac{^{136}\text{Xe}_t}{^{132}\text{Xe}_t} &= \frac{^{136}\text{Xe}_f}{^{132}\text{Xe}_t} + a
 \end{aligned}$$

where the subscripts f, m, a, and t identify the fissiogenic, mantle, atmospheric, and total components respectively, and the a represents the $(^{136}\text{Xe}/^{132}\text{Xe})_a$ ratio. The ratio $(^{136}\text{Xe}/^{132}\text{Xe})_m$ is assumed identical to a ; but if it were identical to that of the planetary primordial component observed in primitive meteorites the error is only 3% and does not affect the conclusion. A least squares fit to the data of Fig. 1 gives a straight line with coefficient of determination $r = 0.97$ and intercept 0.329, identical within limits of error with a . The data therefore indicate mixing of a constant fissiogenic component with varying amounts of atmospheric xenon. The slope of the line gives the concentration of $^{136}\text{Xe}_f \sim 6 \times 10^{-15}$ scc g $^{-1}$ (scc, standard cubic centimetre). $^{136}\text{Xe}_t$ can also be calculated for the individual samples; these values are shown in Table 1.

The He in these samples is overwhelmingly radiogenic. The primordial $^4\text{He}/^3\text{He}$ ratio measured in gas-rich meteorites is $\sim 3,500$. ^3He has not been measured in the samples considered here, but the highest concentration measured in deep-sea glasses is about 90×10^{-12} scc g $^{-1}$ (refs 8, 10), and therefore $^4\text{He}_p < 30 \times 10^{-8}$ scc g $^{-1}$, compared to the total measured values of $1,000\text{--}2,000 \times 10^{-8}$.

The resulting $^4\text{He}_r/^{136}\text{Xe}_t$ ratios are $> 2 \times 10^9$ for all three glasses (Table 1). This is nearly an order of magnitude greater than the production ratio, supporting Alexander's suggestion of mass fractionation due to preferential upward migration of the lighter elements in the rising magma^{22,23}.

In this argument I have attributed the excess ^{136}Xe to spontaneous fission of ^{238}U . All the isotopes except ^{136}Xe are within limits of error of the atmospheric abundances, leading naturally to the supposition that it is something particular about ^{136}Xe that leads to its enrichment; yet the limits of error do not preclude some contribution from an alternative mechanism of isotopic enrichment of the heaviest xenon isotope, such as a mass fractionation process. Nevertheless, my conclusions remain valid despite any such possible complications. The low concentration of 'fissiogenic' xenon gives a high $^4\text{He}_r/^{136}\text{Xe}_t$ ratio, which indicates ^4He enrichment by fractionation of the lighter gas. If part of the excess ^{136}Xe attributed to a fissiogenic origin is actually due to mass fractionation (or to any other process) then the true concentration of fissiogenic xenon is correspondingly lower, the $^4\text{He}_r/^{136}\text{Xe}_t$ ratio correspondingly higher, and this only strengthens the argument that the light gases are enriched by the fractionation process. In turn this conclusion makes the possibility of the xenon being fractionated in such a manner as to enrich the heavier isotopes unlikely, thus leading back to the

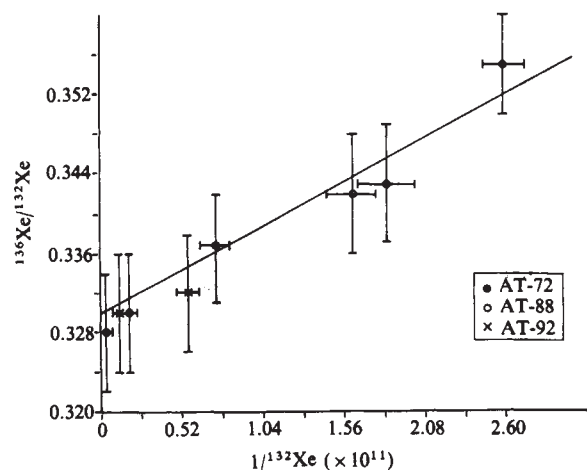


Fig. 1 Xenon isotopic ratios plotted against $1/^{132}\text{Xe}$ for the glasses.

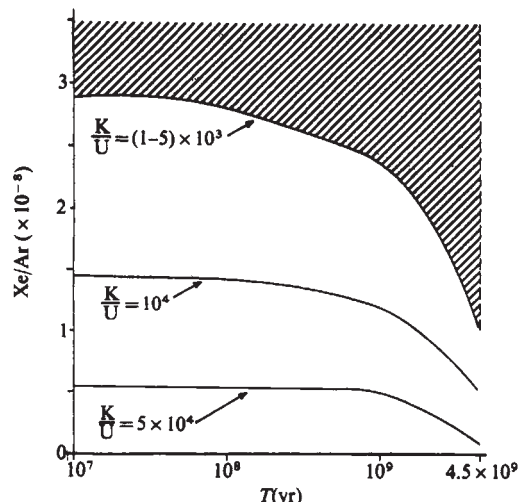


Fig. 2 The $^{136}\text{Xe}_t/^{40}\text{Ar}_t$ production ratio as a function of time.

conclusion that the excess ^{136}Xe is indeed fissiogenic. Therefore even if part of the measured excess ^{136}Xe is not fissiogenic, or if the magnitude of the excess has been overestimated, the conclusion that elemental fractionation of the rare gases has occurred remains valid; that is, the lighter-mass gases are preferentially enriched in these samples.

Previous arguments^{24,25} assessing the terrestrial degassing history and/or the K/U ratio in the basalt source magma, based on the high $^4\text{He}_t/^{40}\text{Ar}_t$ ratio in these rocks, are now seen to be invalid as the He/Ar ratio must also have been enriched by this mechanism. It is not possible from these data to estimate quantitatively the effect on the He/Ar ratio, but we can use both the $^4\text{He}_t/^{40}\text{Ar}_t$ and $^{136}\text{Xe}_t/^{40}\text{Ar}_t$ ratios to set upper and lower limits to the K/U ratio. Although the results have no meaning for the present set of data, the calculation may be useful in future. The production ratio $^{136}\text{Xe}_t/^{40}\text{Ar}_t$ is shown in Fig. 2, the production ratio $^4\text{He}_t/^{40}\text{Ar}_t$ has the same form (see Fig. 1 of ref. 24). The age dependency can be eliminated by considering the ratio $^{136}\text{Xe}_t/\text{U}_m$. The uranium concentration of the oceanic upper mantle (U_m) has been estimated at >20 parts per 10^9 (ref. 26), giving

$$^{136}\text{Xe}_t/\text{U}_m < 3 \times 10^{-7} \text{ ssc g}^{-1}$$

and an age for accumulation of the rare gases $T < 4 \times 10^8$ yr. This is an upper limit both because of the U limit and because the concentration of $^{136}\text{Xe}_t$ may have been enriched over the mantle value in these samples by the same process that led to enrichment of the He/Xe ratio. This age refers not to emplacement of the basalt on the sea floor but to accumulation of the radiogenic gases in the magma. The $^{136}\text{Xe}_t/^{40}\text{Ar}_t$ data of Table 1, compared to Fig. 2 for $T < 4 \times 10^8$ yr, specify an upper limit of $\text{K}/\text{U} < 5 \times 10^4$; the $^4\text{He}_t/^{40}\text{Ar}_t$ data, compared to the calculated production ratio (Fig. 1 of ref. 24) provides a lower limit of $\text{K}/\text{U} > 3 \times 10^3$. These limits (which refer to the basalt source region) encompass nearly the total range of previous suggestions for the deep-Earth K/U ratio.

One other previous attempt to identify the mantle K/U ratio through rare gas data can be faulted by a similar analysis. Hennecke and Manuel measured a low $^4\text{He}_t/^{40}\text{Ar}_t$ ratio on a Hawaiian xenolith¹², concluding that this indicated generation from a K/U source of chondritic composition ($\text{K}/\text{U} \sim 5 \times 10^4$). Their measured Xe displays a small excess at mass = 136 which can be identified as $4 \times 10^{-15} < ^{136}\text{Xe}_t < 24 \times 10^{-15} \text{ ssc g}^{-1}$; the $^4\text{He} = 35 \pm 4 \times 10^{-8} \text{ ssc g}^{-1}$, so that $^4\text{He}_t/^{136}\text{Xe}_t < 9 \times 10^3$, which indicates preferential loss of He. Therefore the low He/Ar ratio may be due to this He loss rather than to a chondritic source. On the other hand, in the Mitchell No. 7 CO_2 gas well of Harding Co., New Mexico, Zartman *et al.* found $^4\text{He} = 45 \times 10^{-6} \text{ cm}^3 \text{ per cm}^3$ (ref. 27), and Butler *et al.* found $^{136}\text{Xe}_t = 9 \times 10^{-14}$ (ref. 17),

giving $^4\text{He}_t/^{136}\text{Xe}_t = 5 \times 10^8$. This result is well within experimental error of the theoretical value 4.2×10^8 and indicates no significant mass fractionation of the radiogenic gases. The measured $^4\text{He}_t/^{40}\text{Ar}_t$ ratio of 1.6 indicates a K/U source of $\sim 1 \times 10^4$, but the source region for these wells probably reflects a crustal rather than a mantle environment.

Deep-sea glasses probably provide a rare gas inventory produced in a mantle environment and not greatly modified by crustal contamination. If a suite of such samples can be found with $^4\text{He}_t/^{136}\text{Xe}_t$ closer to the production ratio, useful limits to the mantle K/U ratio may yet be provided. If, on the other hand, we are able to specify the mantle K/U ratio independently, then measurements of the ratios $^4\text{He}_t/^{40}\text{Ar}_t$ and $^4\text{He}_t/^{136}\text{Xe}_t$ may provide a quantitative estimate of the magmatic mass fractionation process. The mantle components of all the rare gases (and of other volatiles) may then be realistically estimated from their measured abundances in deep-sea glasses.

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Refractory element fractionations among carbonaceous chondrite groups

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Chondritic meteorites consist of collections of grains formed in the solar nebula. Chondritic materials can often be resolved into components that formed in differing nebular conditions. Carbonaceous chondrites are often accorded special significance in planetary models, especially because abundances of most elements in the CI group are indistinguishable from those in the Sun. Abundances of refractory lithophiles are among the most useful parameters for the classification of chondrites, because they systematically decrease through the sequence carbonaceous→ordinary→enstatite^{1,2}. They are also the most general parameter linking groups of very similar chondrites into clans which consist of groups having certain common properties that may indicate formation within a narrow range of distances from the Sun³. We report here newly discovered differences in refractory abundances among the different groups of carbonaceous chondrites.

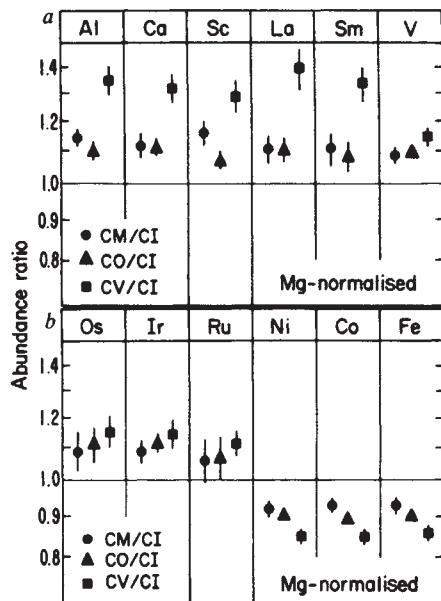


Fig. 1 Mean CI-normalised atomic abundance ratios for; a, refractory lithophiles; and b, refractory and non-volatile siderophiles. Uncertainty limits are 80% confidence limits. Our CI abundances (Mg normalised, Mg = 100) are: Al = 8.16, Ca = 5.87, Fe = 82.4, Co = 0.219, Ni = 4.48, Sc = 3.26×10^{-3} , V = 2.78×10^{-4} , La = 4.17×10^{-5} , Sm = 2.40×10^{-5} , Ru = 1.69×10^{-5} , Os = 6.34×10^{-5} , Ir = 5.49×10^{-5} .

Carbonaceous chondrites are divided into four groups, CI (high volatile abundances, no chondrules), CM (intermediate volatile abundances, small (~0.2 mm) chondrules), CO (low volatile abundances, small chondrules) and CV (low volatile abundances, large (~1 mm) chondrules). CV and CO chondrites were thought to belong to the same group, until Van Schmus^{4,5} pointed out textural differences and McCarthy and Ahrens⁶ found higher refractory lithophile abundances in CV than in CO. In the past it was believed that refractory lithophile abundances are indistinguishable in CI, CM and CO (refs 4, 5), and refractory siderophiles indistinguishable in all four carbonaceous groups^{7,8}. In fact, our neutron activation data show that the carbonaceous groups can be divided into CI, CM-CO and CV clans based on differences in refractory lithophile abundances, and that refractory siderophiles do not parallel refractory lithophiles.

Nineteen carbonaceous chondrites were analysed: two CI chondrites (Orgueil, Alais), nine CM chondrites (Murray, Murchison, Mighei, Nogoya, Santa Cruz, Cold Bokkeveld, Yamato 74662, Cochabamba, Allan Hills A77306), five CO chondrites (Lancé, Felix, Ornans, Kainsaz, Warrenton) and three CV chondrites (Allende, Mokoia, Vigarano). Two samples (~300 mg each) of each meteorite were analysed in separate irradiations, with the exception of Orgueil (four samples), Allende (six samples), Santa Cruz (one sample) and Allan Hills A77306 (one sample). Among the elemental concentrations determined were several refractory lithophiles (Ca, Al, Sc, La, Sm), refractory siderophiles (Os, Ir, Ru) and a suitable normalising element (Mg). Complete data for some 25 elements will be published elsewhere.

The common practice of combining elemental concentration data from sources that may include systematic biases can unduly 'broaden' the abundance ranges. One such bias can result from the variable state of hydration of CI and CM chondrites. Producing our own large data base for each carbonaceous chondrite group and determining a suitable normalising element in the same sample enabled us to avoid these problems and has resulted in a clearer picture of elemental abundance variations among the different groups.

Refractory lithophile abundances in CM, CO and CV chondrites relative to CI are plotted in Fig. 1a. The data are Mg-

normalised as we do not determine Si. Normalising to Si would not noticeably change the plots, as the Mg/Si atom ratio is essentially identical for CI, CM and CO and at most 3% higher for CV⁶. Our results confirm the observation⁶ that CV refractory lithophile abundances are considerably higher (~ $\times 1.34$) than those in CI. The higher abundance of refractories in CV chondrites may result from an enhancement of the large refractory inclusions present in this group relative to the other components (matrix and chondrules) carrying the major lithophiles Si and Mg. The higher abundance of refractory inclusions is attributed to differential settling of grains to the median nebular plane⁹, with the rate of settling being proportional to the density and radius of the grains. At the median plane grains could agglomerate to planetesimals by gravitational collapse^{9,10}. Mg concentrations in the refractory inclusions in Allende are lower than those in the whole rock; V concentrations^{11,12} are higher, but inclusion/CI ratios are only about half as high as values found for highly refractory elements^{12,13}, thus accounting for the lower CV enhancement ($\times 1.14$) of V.

Our most unexpected discovery was that CM and CO refractory lithophile abundances are distinctly higher, by ~1.12 and ~1.09, respectively, than those in CI chondrites. Oxygen isotope data¹⁴ are not inconsistent with an interpretation that the ~10% difference in refractory contents between CM-CO and CI indicate formation at different nebular locations. CI chondrites and CM matrix scatter along fractionation lines differing about 3% in $^{17}\text{O}/^{16}\text{O}$ ratio when extrapolated to the same $^{18}\text{O}/^{16}\text{O}$ ratio. The CI results are whole rock data and probably reflect equilibration with nebular gases. It may be that the CM data result from equilibration inside a parent body, but if they also reflect nebular equilibration, the differing O isotope compositions indicate different formation locations for CM and CI.

The rare refractory-rich inclusions in CM and CO chondrites¹⁵⁻¹⁷ are smaller (~0.1 mm) than those typical of CV chondrites (~1 mm). As refractory inclusions are unknown in CI chondrites, the presence of these in CM and CO could possibly account for the small refractory lithophile enrichment. McSweeney¹⁸ reports that anhydrous inclusions are about 2.5 times more abundant in CO than CM chondrites. If the refractory fraction of anhydrous materials is the same in each group and our simple model is correct, the abundance of refractory-rich materials should be about the same in CM and CO. However, CM refractory inclusions apparently have higher refractory contents than CO inclusions¹⁷, and part of the

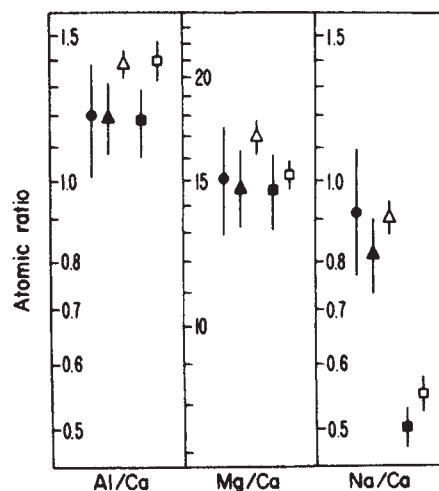


Fig. 2 Element/Ca ratios for CI and CM carbonaceous chondrites and the Sun from this work (Δ , CI; $\square$, CM) and from compilations of Holweger²⁴ ($\bullet$, solar; $\blacktriangle$, CI; $\blacksquare$, CM). Our CM Mg/Ca ratio is closer to the solar ratio than our CI value, but our Na/Ca value, like Holweger's is much lower than the reported solar ratio.

discrepancy may also have resulted from aqueous alteration and destruction of some CM inclusions^{18,19}.

It thus seems best to divide the carbonaceous chondrites into three separate clans: CI, CM-CO and CV chondrites. The refractory lithophile data are inconsistent with the formation of CI, CM and CO chondrites from two rigidly defined components (matrix + anhydrous minerals including chondrules)²⁰.

Figure 1b shows Mg-normalised refractory siderophile abundances for CM, CO and CV relative to CI. Also plotted are the siderophiles Fe, Ni and Co that are neither refractory or volatile. Refractory siderophiles are 1.06 to 1.15 times higher in CM, CO and CV than in CI. Previous analyses^{7,8} have not revealed this enrichment, perhaps partly as a result of the lack of internal normalisation and partly by the sometimes seemingly arbitrary selection of mean CI concentrations. Morgan and Lovering²¹ did note a small enhancement of Os and Re in CM relative to CI but attributed it to probable sampling errors. Refractory siderophile abundance ratios seem to decrease as CV > CO > CM. In each group Os and Ir ratios are about the same whereas Ru values are slightly smaller. This may be related to the fact that Ru is less refractory²². Relative to Os and Ir, CI-normalised Ru is less abundant in one refractory inclusion¹³ and in 50 metal particles from another inclusion²³ of the Allende CV chondrite. Excluding Ru, abundance ratios of the refractory siderophiles in CM and CO chondrites are approximately the same as those of refractory lithophiles within analytical error, whereas in CV refractory siderophiles are about 15% lower than refractory lithophiles.

Refractory siderophiles are on average less abundant than refractory lithophiles in type B and coarse-grained refractory inclusions from Allende²⁴, and the siderophile/lithophile ratio is still lower in fine-grained inclusions. This suggests why refractory siderophiles are not as enriched as refractory lithophiles in CV chondrites. If a significant fraction of the refractory siderophiles was present in other kinds of carrier grains, these grains may also have been the dominant carriers of the common siderophiles (Fe, Co, Ni) that are nearly 15% deficient in CV relative to CI (Fig. 1b). We recently analysed a fine-grained chondritic inclusion from Allende²⁵ and found it to be enriched in refractory and common siderophiles, but depleted in refractory lithophiles relative to CV chondrites. The inefficient settling to the nebular midplane of similar fine-grained material could account for the observed siderophile/lithophile fractionation at the CV formation location.

On the other hand, the lower abundance ratios of Fe, Ni and Co in CM ($\times 0.93$) and CO ($\times 0.90$) relative to CI is not paralleled by a resolvable decrease in the abundance of refractory siderophiles relative to refractory lithophiles, suggesting that the carrier of the lost Fe, Ni and Co in CM and CO chondrites must have had much lower refractory siderophile/Ni ratios than the siderophile carrier lost during the formation of CV chondrites. Thus the carriers of both refractory siderophiles and refractory lithophiles were different at the CV location from those at the CM-CO location.

The actual nebular processes were probably more complex than those outlined here. Instead of two (one refractory, one nonrefractory) lithophile and two siderophile components there was probably a continuum of compositions which we have roughly approximated by a four-component model. It would seem that the four groups of carbonaceous chondrites are not as closely and as simply related as earlier thought, but are distinguished by chemical and isotopic fractionations suggesting their formation in differing conditions, and probably at differing distances from the Sun.

Recently, Holweger²⁶ examined the compositional relationship between the solar photosphere and chondrites to determine which chondrite group best approximated the solar composition. His work included five elements (Na, Mg, Al, S, Ca) determined to precisions of about $\pm 10\%$ in the Sun. Because of our improved data base, and because some chondrites were incorrectly classified in Holweger's data source, it seemed worthwhile to repeat the comparison. We do not analyse for S,

so it is not included. In Fig. 2 Holweger's CI, CM and solar ratios of Al, Mg and Na to Ca are compared with our chondrite data. Our Al/Ca ratios are higher than Holweger's, but agree well with X-ray fluorescence data⁶. Holweger's conclusion that CI chondrites closely resemble the solar composition is also supported by our data. All CI, CM and solar ratios for non-volatiles agree within experimental error, but the CM ratio for the volatile Na is almost half the reported solar ratio. However, some other chondrite groups have higher Na abundances approaching CI values, and accurate solar data on additional volatile elements are needed before the CI-solar congruence can be verified.

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Radiocarbon dating evidence on the impact of atmospheric pollution on upland peats

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When radiocarbon dating is applied to young sample materials, the results are subject to the Suess effect¹: that is, the concentration of atmospheric ¹⁴C has been diluted by carbon dioxide containing negligible ¹⁴C from the burning of fossil fuels since the onset of the industrial revolution. As this carbon dioxide is incorporated into growing plants in the normal manner, radiocarbon dating of young plant material tends to produce spuriously old dates. The age discrepancy could, in theory, be as much as 250 yr (ref. 2). However, during a study into the age, origin and vegetational history of upland blanket peats in South Wales, substantially older dates were obtained from recent horizons. The dating of different peat fractions by radiocarbon assay suggested that the substantially older dates derived from contamination by particulate pollution during the industrial revolution. The scale and impact of this pollution are discussed in the context of these results.

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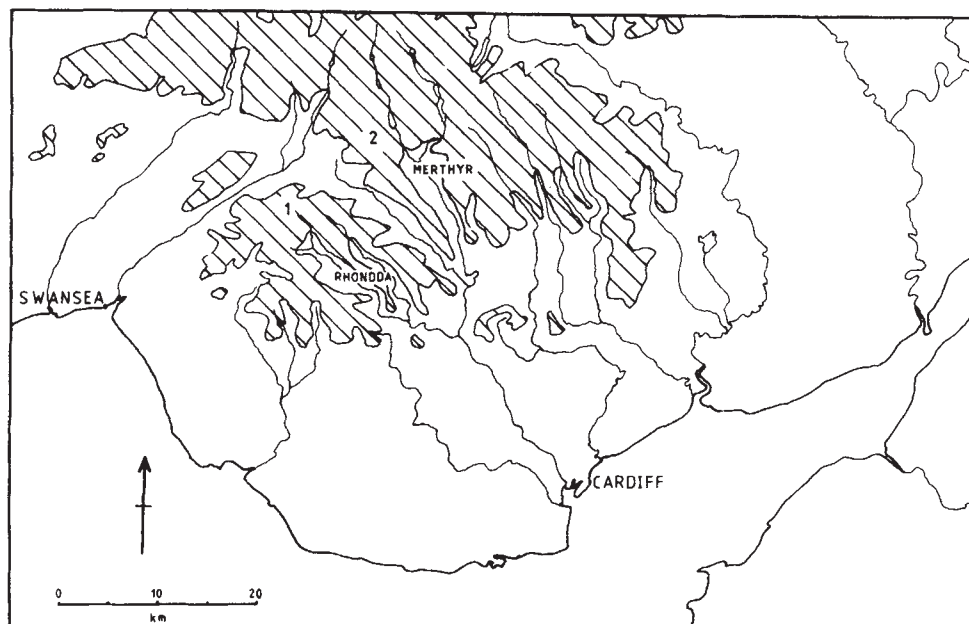


Fig. 1 Location of sites on map of South Wales: 1, Cefn Ffordd; 2, Coed Taf. Land over 300 m OD shown.

The blanket peats of upland South Wales are widespread and often shallow. Two profiles with less than 40 cm peat accumulation were investigated by pollen analysis, the sites being in close proximity to the industrial Rhondda and Merthyr valleys: Cefn Ffordd (SN 906032, 600 m OD) and Coed Taf (SN 988108, ~400 m OD) (see Fig. 1). In pollen diagrams from both sites, pollen spectra in the upper horizons showed a distinct change from those characterised by high *Ericaceae* values (mainly *Calluna vulgaris*—heather or ling) to a pronounced dominance of *Gramineae* (grass) pollen (Fig. 2). This seemed to record a shift from *Callunetum* to the present vegetation in which *Molinia caerulea* (purple moor grass) is dominant (Cefn Ffordd is now afforested with spruce). To date this and other changes, peat samples were prepared for radiocarbon dating using a pretreatment designed to remove those components likely to give an underestimate of the true radiocarbon age of the peat. The potentially more mobile 'humic acid' fraction was extracted in two alkali washes, and contamination from modern rootlets was minimised by passing the sample through a 250- μ m mesh sieve. Dates were obtained on the resultant fine particulate fraction (F) as Dresser³ had previously found this gave the most reliable estimate of the true radiocarbon age of blanket peat samples.

The pretreated samples were burnt, and after purification, the resultant carbon dioxide was mixed with hydrogen, passed over a ruthenium catalyst and converted into methane. The ^{14}C activity of the methane was measured at a pressure of 2.5 bar in a conventional 2-l copper gas proportional counter which was surrounded by a multi-anode anticoincidence counter and 8 ton of pre-nuclear age steel. Quoted precisions of the dates are one standard deviation and derive mainly from counting statistics.

The accumulation rate of blanket peats is variable⁴: at depth, the rate may be of the order of 50–100 yr cm^{-1} , but towards the surface where autocompaction is not as intense and the peat less humified, the accumulation rate may seem greater. Microscopic examination of the Cefn Ffordd profile revealed particles of coal dust in the upper 2 cm thought to correspond with modern coalmining. From these two lines of evidence a rough estimate of 100–250 yr BP was made for the inferred heather moorland-grassland transition. However, the radiocarbon date for a sample at 4–5 cm was $2,970 \pm 50$ yr BP (CAR-47), some 2,000 yr older than a sample from 8 to 10 cm which was dated to 925 ± 45 yr BP (CAR-106).

Samples from the Coed Taf site were dated subsequently using the same pretreatment. In the upper 2 cm, particles akin to soot were detected but no coal dust was observed. A date of 140 ± 50 yr BP (CAR-81; $1,810 \pm 50$ yr AD (ref. 5)) was obtained

for a sample at 11–13 cm, which strongly suggested that the peat of a sample at 6–8 cm would have accumulated after the start of the industrial revolution in Wales. The radiocarbon age of this sample was $3,710 \pm 90$ yr BP (CAR-82F).

Thus at two sites, 11-km apart, upper horizons of peat produced radiocarbon dates that were apparently too old. Both samples pre-date similar changes in pollen spectra from which one might infer a similar vegetational change.

These old dates needed to be explained. Both were checked. Laboratory contamination could almost certainly be ruled out as contamination during pretreatment would be more likely to introduce modern (more radioactive) than older carbon.

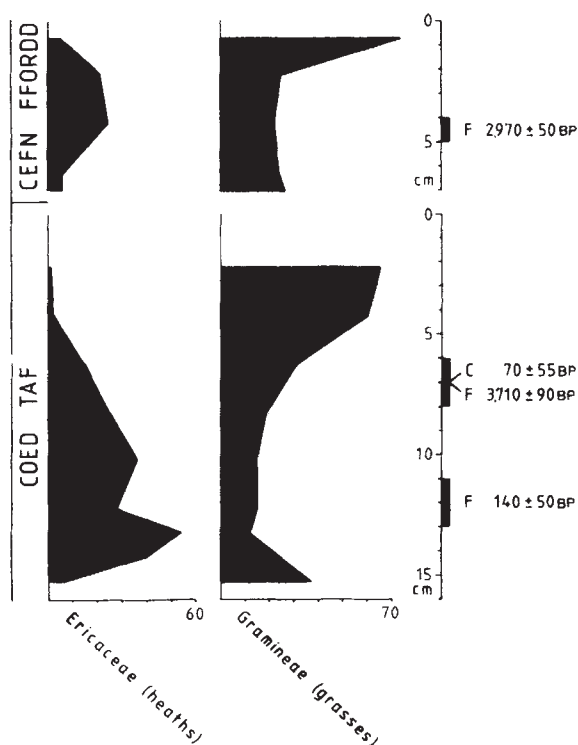


Fig. 2 *Ericaceae* and *Gramineae* curves (percentage total land pollen) for surface horizons of Cefn Ffordd and Coed Taf. Radiocarbon dates on humic acid (C) and fine particulate (F) fractions shown. For laboratory code numbers, see text.

Contamination during peat accumulation by material derived from older layers must, however, be considered. Eroding peat can be redeposited by water or wind. Either explanation is improbable because: (1) gross contamination from older peats would have been required to produce dates so old. Redeposition on this scale can be fairly easily recognised in the field but was not observed at either site. (2) Even the basal peat at Coed Taf is markedly younger than the date obtained for the fine particulate material at 6–8 cm. (3) Both sites were deliberately selected as being on convexities where inwash would be minimal. (4) There seem to be no recently eroded areas nearby that could have produced either windblown or waterborne organic material.

The incorporation of ancient carbon into a sample would dilute the activity of contemporary carbon, but there was no visual evidence of coal dust within the horizons dated. Although soot was used as a fertiliser during the nineteenth century, this seems to have been restricted to areas of market gardening or for improving heavy clay soils; its use on moorland in South Wales is not mentioned by Osborne⁶. Incompletely burnt fossil fuel (such as smoke and smut), carried up above the industrial valleys and deposited or rained out onto the moorland⁷, seemed to be the only feasible source of old carbon.

This hypothesis was tested by dating the humic acid component of the 6–8-cm sample from Coed Taf. If the peat were as old as the F-fraction date had suggested, then the humic acid (C-fraction) would be expected to be of comparable age, or slightly younger^{3,8}. The date obtained was considerably younger at 70 ± 55 yr BP (CAR-82C). This result could be obtained only from organic material produced after the start of the industrial revolution.

The implications of these results may now be considered.

(1) Atmospheric pollution produced by the substantially increased exploitation of coal during the industrial revolution has been incorporated in these South Wales upland peats. The discovery of this by radiocarbon dating enables some quantitative estimate of the scale of pollution to be made. Carbon from coal contains no detectable ^{14}C . In order for the fine particles of true age 100 yr BP to produce such old ^{14}C dates, more than 30% contamination of the organic carbon fraction by inactive carbon would be required. Assuming the apparent increase in age was due to the incorporation of inactive carbon, this would seem to comprise $36.4 \pm 0.8\%$ of the carbon content of the fine fraction of the Coed Taf 6–8-cm sample. Based on data from the combustion of the F-fraction in the dating process, and assuming that all the inert carbon was contained in this component, the original (untreated) peat sample contained 0.83 ± 0.01 g inactive carbon. This sample had an area of ~ 64 cm² and was accumulated over ~ 32 yr (based on the peat accumulation rate). These figures indicate there was an inactive carbon deposition rate of the order of 4 g yr⁻¹ m⁻². (The original work did not envisage this type of treatment, but a subjective appraisal of the errors involved indicates an error of ± 2 g yr⁻¹ m⁻²). The Cefn Ffordd inactive carbon deposition rate was of the order of 2.5 g yr⁻¹ m⁻².

(2) In the radiocarbon dating of recent moorland vegetational changes near industrial areas, the use of unpretreated peat, or certain components, is likely to produce an erroneous result. Further research is still required, however, to ascertain the reliability of dates on the humic acid fraction as this component of blanket peats has already been shown to yield dates which are slightly too young⁸.

(3) The transition from heather moorland to *Molinia* moor seems to have taken place at the two sites after the start of the industrial revolution. Did, then, changing moorland management practices associated with disrupted traditional employment patterns bring about such a marked vegetational change, or was the recent degeneration of heather moorland in some way connected with airborne pollution? Conway was inclined to discount the influence of atmospheric pollution on Pennine bogs⁹, but the scale may not have been fully appreciated: deposition of some 0.6–1.8 ton per hectare inactive carbon over a 30-yr time-span is indicated for these South Wales moorlands.

These figures convert to between 5.5 and 16.5 mg m⁻² d⁻¹ when expressed in the form used for modern deposit-gauge observations on undissolved deposits and ash yields¹⁰.

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Agnathans from the Devonian of China

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Our Anglo-Chinese re-examination of material from southern China has led to a reappraisal of the two major groups of the primitive armoured jawless fish, the ostracoderms. We have united the galeaspid and polybranchiaspids in the Galeaspida of equal rank to the Osteostraci (Cephalaspides) and grouped them within the same major agnathan division as the anaspids and lampreys—the Cephalaspidomorphi. We also now believe that during the Lower Devonian, southern China was part of an isolated faunal province in which the agnathans experienced a major evolutionary radiation, independently from the rest of the world.

Fossil agnathans were first listed from southern China in 1937 by Ting and Wang¹ and, during the International Geological Congress in 1948, Young² reported rich deposits of Devonian vertebrates containing the agnathan *Cephalaspis*. This material was never described and its whereabouts are unknown. However, work in the same area in the same horizons in the early 1960s yielded several unusual forms³. The new genera *Galeaspis* (Fig. 1) and *Nanpanaspis* (Fig. 3b) were assigned to the Osteostraci (Cephalaspides), while *Polybranchiaspis* (Fig. 2) was placed in the Heterostraci (Pteraspides).

These fossils were unlike any previously described agnathans and their relationships to the known major groupings became a subject of controversy. It was suggested that they represented a new group of Agnatha to which the name Galeaspida was given^{4,5}. This view received support from Stensio⁶ and Janvier⁷ but was rejected by Miles⁸. Novitskaya^{9,10} considered that the polybranchiaspids possessed a combination of osteostracan and heterostracan features and hence concluded that there was "no complete morphological break" between these two groups.

In the 1970s many new agnathan genera were described^{11–14}, including *Laxaspis*, *Sanqiaspis*, *Huanaspis*, *Asiaspis* and *Lungmenshanaspis* (Fig. 3), all of which were classified with the heterostracans. P'an¹³ erected a new order of Heterostraci, on the basis of fragmentary remains of the genus *Hanyangaspis*. (This genus has been restudied and two of us (L.B.H. and Y.-H.L.) consider that it can be interpreted as a polybranchiaspid¹⁵.)

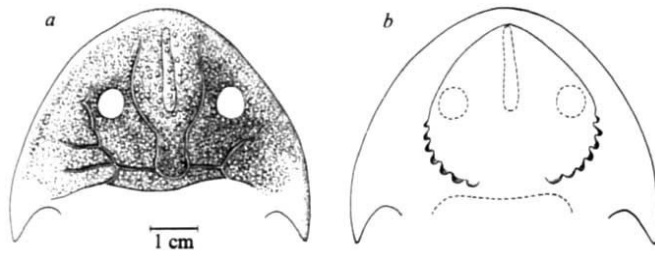


Fig. 1 Restoration of *Galeaspis* based on holotypes of *G. changi* Liu and *G. xujiaichongensis* Liu. *a*, Dorsal view showing median dorsal opening, orbits, posterolateral spines and sensory canal system. *b*, Ventral view showing branchial openings and outline of buccal floor (IVPP, Peking).

P'an and Wang¹⁴ identified the new genus (*Nakaolinaspis*) as a member of the amphiaspid heterostracans, a group confined to the Devonian of Siberia¹⁶. A restudy of this genus has convinced us that it was based on part of the skull roof of a specialised placoderm and hence there is no evidence that the amphiaspids are represented among the Chinese agnathans.

Southern China was clearly a province where agnathans underwent a major radiation. They were different from the familiar groups known from other parts of the world and their structure and relationships were controversial. We have re-examined the material in the Geology Museum and Institute of Vertebrate Palaeontology and Palaeoanthropology in Peking and reappraised the relationships.

The galeaspids (s.s.) and polybranchiaspids both possess a bony carapace covering the anterior part of the body, both dorsally and laterally. The lateral margin forms a longitudinal carina which extends on to the ventral surface and may be drawn out to form posteriorly or laterally directed spines. Similarly the anterior margin of the carapace forms an anterior ventral rim which is continuous with the lateral margin and may also be drawn out into an anterior projection or rostral spine.

The dorsal surface of the carapace is perforated by three openings—towards the anterior margin, a pair of circular openings which may be dorsally or laterally placed, and a median opening which may be transversely oval (*Polybranchiaspis*), circular (*Duyunaspis*), arcuate and transversely elongated (*Sanqiaspis*) or narrow and longitudinal (*Galeaspis*). On the inner surface of the armour there is a small round depression behind the dorsal median opening, which does not penetrate the carapace.

The inner margin of the ventral rim forms a smooth arc which runs posteriorly to the level of the paired circular perforations, behind which level the ventral rim at its median border forms a

series of smooth notches. In some specimens small oval or circular plates are preserved adjacent to these notches and partially covering them.

The central median dorsal opening has a rim of fine ornament on its inner margin and in well preserved specimens the opening is raised somewhat above the general level of the rest of the carapace. In many specimens there is a well demarcated moat-like groove in front of the anterior margin of the opening. In a few specimens of *Polybranchiaspis* it can be seen that this opening was covered in life by an oval plate (Fig. 4). (Studies of photographs by J. A. Halstead and L.B.H. have revealed the presence of a similar plate in *Galeaspis*.) Behind the carapace in some specimens the squamation is preserved. On the ventral surface of the tail there are small diamond-shaped scales, the lateral scales are narrow and deep; in some forms there are dorsal ridge scales. There is no evidence of movable paired appendages and in *Sanqiaspis* it can be demonstrated that the tail was reversed heterocercal or hypocercal.

The sensory canal system comprises a similar pattern, as can be seen in Figs 1 and 2. The preservation is also comparable in

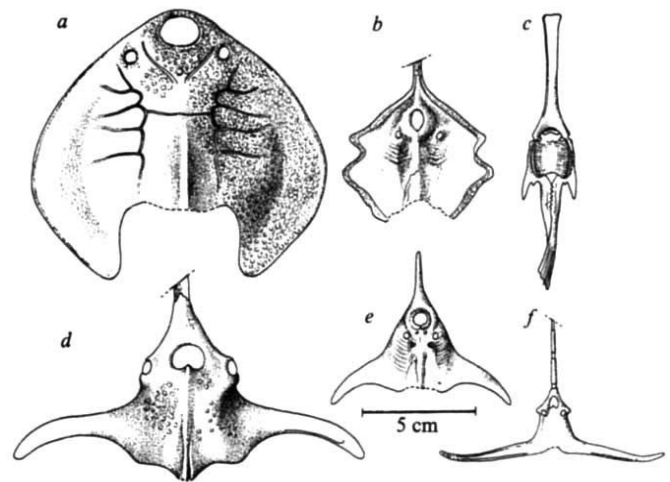


Fig. 3 Galeaspid genera in dorsal view to illustrate variety of external form. *a*, *Laxaspis* Liu; *b*, *Nanpanaspis* Liu; *c*, *Sanqiaspis* Liu; *d*, *Huanaspis* Liu (IVPP Peking); *e*, *Asiaspis* P'an; *f*, *Lungmenshanaspis* P'an and Wang (Geological Museum, Peking).

both groups, in that the canals are enclosed within the armour. Frequently, as in *Polybranchiaspis*, they appear to lie in grooves on the surface, but in well preserved specimens they are covered by the superficial ornament (Figs 2 and 4b).

Many of the morphological features dealt with here have never been the subject of disagreement. It is universally accepted that the paired circular dorsal or lateral perforations of the carapace must be the orbits, while the small rounded depression, behind the dorsal median opening, is the position of the pineal organ. Similarly the notches on the median margin of the ventral part of the lateral extension of the dorsal carapace represent the individual openings of the gills or gill pouches.

The most controversial structure is the dorsal median opening, previously interpreted as a nasohypophyseal opening in *Galeaspis* and a mouth in *Polybranchiaspis*. The size of the opening seems to militate against it being nasohypophyseal, yet the position and profile seem inappropriate for a mouth. The discovery of a cover plate *in situ* in *Polybranchiaspis* and *Galeaspis* (Fig. 4) shows that it could not have functioned as a mouth and hence the inner margin of the ventral rim must have marked the anterior margin of the mouth, as in other agnathans such as cephalaspids and heterostracans. We suggest that the dorsal opening housed a sensory organ concerned with olfaction. There is no evidence as to whether this structure served as an inlet for water into the buccal cavity and hence to the gills, as a kind of analogue of the spiracle found in amphiaspid heterostracans and living skates and rays. Certainly for a benthonic

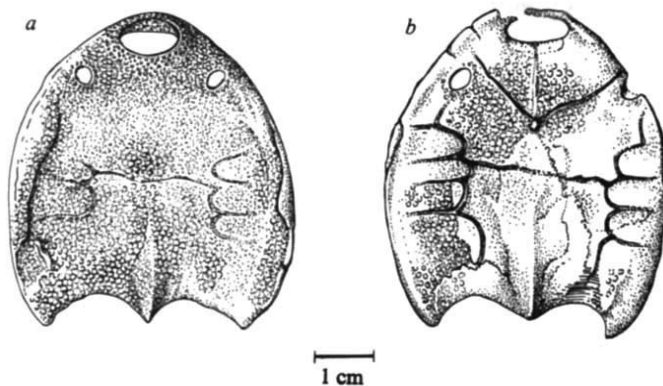


Fig. 2 *Polybranchiaspis liaojiaoshensis* Liu. *a*, Dorsal view showing median dorsal opening and dorsal orbits, with superficial ornamentation covering sensory canal system. *b*, Holotype, in dorsal view with outer surface removed to expose underlying sensory canal system (IVPP, Peking).

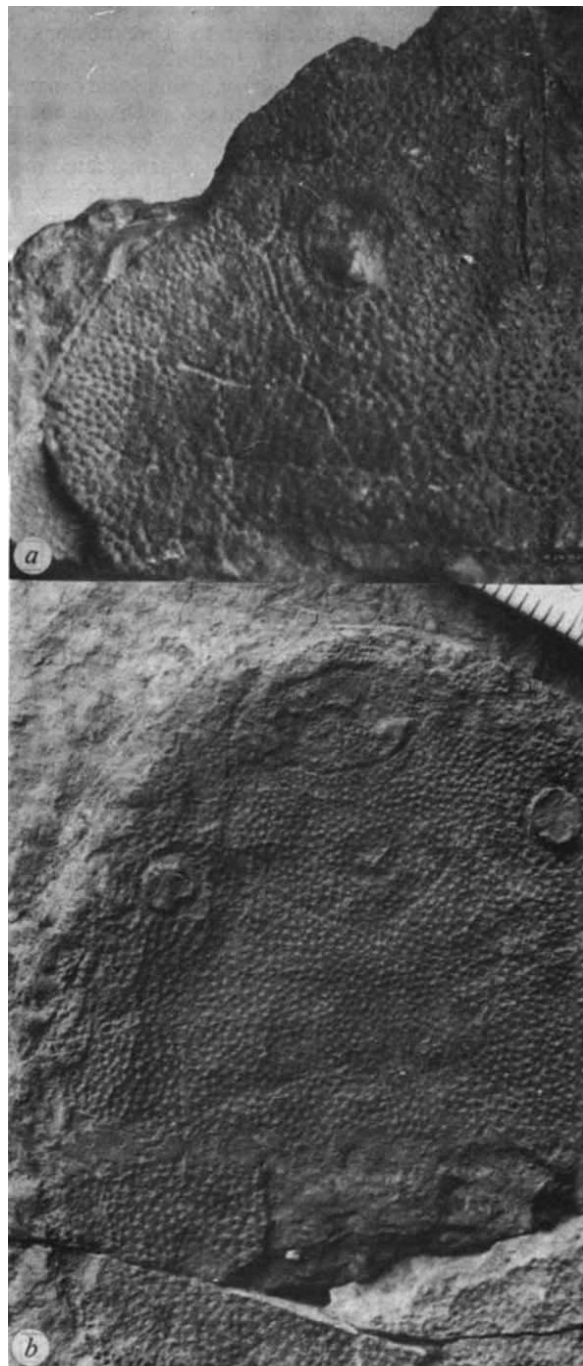


Fig. 4 a, *Galeaspis changi* Liu, holotype, dorsal view showing cover plate in position over median dorsal opening (IVPP, Peking). b, *Polybranchiaspis liaojiaoshensis* Liu, dorsal view showing cover plate in position over central median dorsal opening (IVPP, Peking).

microphagous feeder, it would make good functional sense for this opening to have served such a purpose.

From our examination of the morphology of the galeaspids (s.s.) and polybranchiaspids and the interpretations of the structures to which we have now been led, we agree that both groups can be united into a single major taxon of high rank, the Galeaspida, equivalent to Osteostraci and Anaspida.

The Galeaspida, Osteostraci and Anaspida all have three main dorsal openings, and separate gill openings. The first two groups have similarly preserved endocranial connective tissue^{12,17}. Features which appear to link the Galeaspida with the Heterostraci include possible double nasal organs, a simple brain, a pineal body covered by the external armour and the

overall shape of the carapace and hypocercal tail. These characters cannot be considered diagnostic as they are all primitive and in some cases would apply to such primitive cephalaspids as the tremataspids. The Chinese agnathans can be excluded from association with the heterostracans but they can be linked in a general way with the cephalaspids.

With the overall systematic position of the galeaspids agreed in the context of the known agnathan groups, it is evident that *Galeaspis* closely resembles the cephalaspids in having its gill openings arranged into a more or less circular pattern and also in having fewer gill openings than do the polybranchiaspids. The latter seem to be less specialised in this regard, with gills arranged serially along the anterior part of the body, although in the overall proportions of the carapace the polybranchiaspids vary greatly. The apparent similarity of *Galeaspis* to the cephalaspids, with the exception of the presence of lateral and posterior dorsal sensory fields, may indicate a degree of direct relationship or parallel evolution as the organisms become adapted for an ecological niche comparable to that occupied elsewhere by the cephalaspids.

The Galeaspida represent a unique major evolutionary radiation of the ostracoderms, which appears on present evidence to have been confined to the south China province. With the possible exception of the Silurian cyathaspid *Kiangsuaspis* described by P'an¹⁸ there is no sign of any agnathan that can be linked to forms known from other parts of the world. The firm impression is that, as far as agnathans were concerned, this region was isolated during the Lower Devonian.

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Internal anatomy of the polybranchiaspids (Agnatha, Galeaspida)

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A number of polybranchiaspids (Agnatha) from the Devonian of China have parts of their soft anatomy, including the central nervous and vascular systems, extremely well preserved as mineral replicas. This has made possible direct comparisons with the internal anatomy of the living agnathans, the hagfish and lampreys, as well as the fossil heterostracans and cephalaspids. The polybranchiaspids do not seem to be related to either the hagfish or the heterostracans, but instead represent a separate grouping, the Galeaspida, allied to the lampreys and cephalaspids.

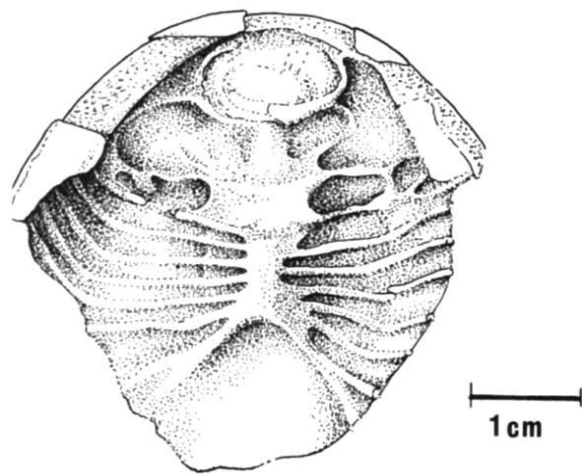


Fig. 1 *Polybranchiaspis liaojiaoshensis* Liu, V.3027, Institute of Vertebrate Palaeontology and Palaeoanthropology, Peking, internal view of perichondral ossification of endocranial tissue showing mouldings produced by orbital sinus and somites.

The anatomy of the polybranchiaspid carapace is now well known¹⁻⁴. The neurocranial floor on the inner surface of the dorsal carapace of *Polybranchiaspis* (Fig. 1) was described by Liu². Beneath the superficial ornamentation at the same level as the sensory canal system, there was a subaponeurotic (subcutaneous) vascular plexus (Fig. 2).

P'an and Wang³ described several polybranchiaspid specimens, in particular *Duyunaspis*, in which after death the spaces occupied by the central nervous and vascular systems were filled by a secondary iron mineral and the bony material had dissolved away to leave a mineral replica of the internal anatomy (Fig. 3).

There are only two pairs of semi-circular canals in the ear, the anterior and posterior vertical, both with clearly defined ampullae. The two canals meet medially as the point where the utricle is situated and from where it develops ventrolaterally. The sacculus, in contrast, is frequently expanded dorsomedially so that it comes to lie medial to the semi-circular canals. The round structure arising from beneath the canals and situated between them and the brain, is best interpreted as the sacculus.

The position of the orbits is in the region labelled 'premandibular elevation' by P'an and Wang³. The eyes are unlikely to be adjacent to the dorsal median opening but rather as in other polybranchiaspid genera¹⁻⁴. The position of the nasal sacs is more difficult to decide. There is no justification for the positioning of the nasal sacs at the anterior margin of the specimen³, a more likely position is immediately lateral to the dorsal median opening. There appears to be a nerve running from the forebrain region to this structure and, on this interpretation, this would be the olfactory nerve. The other possibility is the two structures immediately behind the dorsal median opening which P'an and Wang³ identified as 'possible nasal lobes'.

The dorsal median opening is transversely oval but a posteriorly directed V at its posterior margin indicates a canal leading to the hypophysis, suggesting that the dorsal median cavity was a nasohypophyseal structure. There is no evidence as to whether this opened directly into the buccal cavity as in hagfish or was blind as in lampreys and cephalaspid.

The infilling of the endocranial cavity indicates the general configuration of the brain. The spinal cord with its accompanying spinal nerves expands anteriorly into the medulla oblongata, the major part of the hindbrain. There is no evidence of a transverse commissure representing the cerebellum or additional lobes relating to the development of the acoustico-lateral system. The constriction lying at the junction of the anterior and posterior semi-circular canals of the ear was the position of the midbrain, even though in higher vertebrates it is always situated anterior to the ear region. In the polybranchiaspids, the pineal organ was sited at the level of the anterior semi-circular canals and therefore the midbrain must have been posterior to it.

The anterior part of the forebrain, the telencephalon, is either represented by the pair of irregular structures laterally or by the region lateral and deep to the pineal. In either interpretation, a degree of cranial flexure occurred as a consequence of the development of the median dorsal organ.

The identification of the cranial nerves that can be observed, presents considerable difficulties and hence must be considered tentative. The two cranial nerves seen on the right running obliquely posterolaterally were probably the different branches of the vagus X, and the nerve anterior to these but posterior to the ear region, the glossopharyngeal IX. Two nerves in close proximity running anterolaterally but diverging somewhat and approaching the brain beneath the semi-circular canals of the ear were the facial VII and trigeminal V nerves.

The most prominent feature of the vascular system was the pair of large calibre vessels running on either side of the labyrinth of the ear and the brain, the *vena capitis lateralis*³ or lateral head vein, anteriorly ending in a large orbital sinus. Posteriorly there was the occipital venous sinus. It is not possible to determine with any certainty at which point the veins from the anterior and posterior parts of the body joined to form the common cardinal vein or *ductus Cuvieri* leading to the heart, and for this reason topographical terms are used. A series of segmental veins leading into the *vena capitis lateralis* are clearly preserved. Lateral to the occipital venous sinuses were further vessels draining segmental structures, paired dorsal aortae, from the efferent arterial system.

The most prominent aspect of the internal anatomy of the polybranchiaspids, observed in most genera^{3,4} were a series of paired (up to 20 in number) dorsally positioned structures rising up on either side of the central nervous system, extending laterally some distance, and then curving posterolaterally and each associated with a separate branchial opening along the ventrolateral margin of the carapace. Such dorsal structures have been interpreted as representing the former position of gill



Fig. 2 *Polybranchiaspis liaojiaoshensis* Liu, portion of subaponeurotic (subcutaneous) vascular plexus and sensory canal system enclosed within the dermal armour.

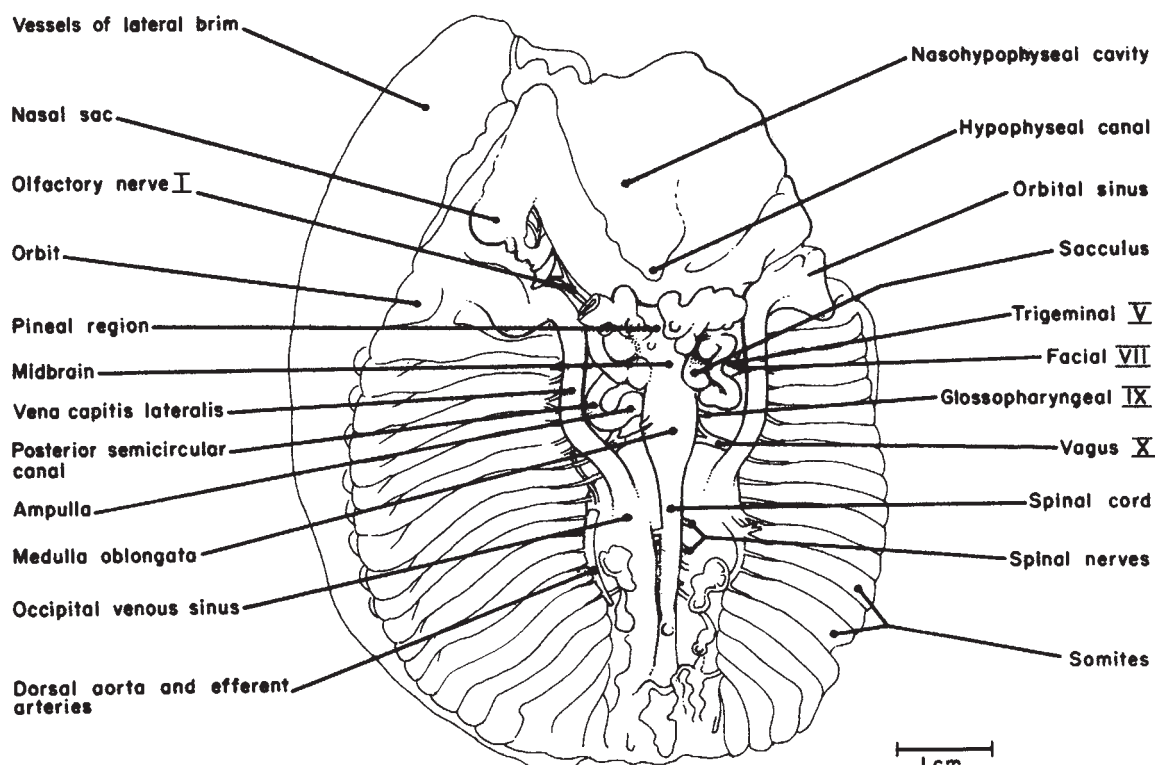
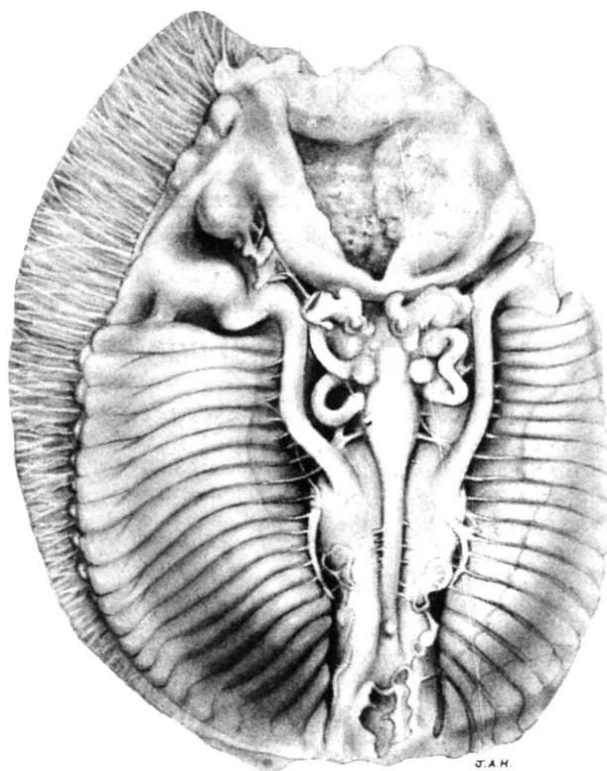


Fig. 3 *Duyunaspis paoyangensis* P'an and Wang, holotype, V.1324, Museum of Geology, Peking, in dorsal view showing mineral infilling of canals and spaces in endocranial connective tissue which have become exposed subsequent to the removal of the original calcified tissues. Identification of structures indicated in accompanying annotated diagram.

pouches^{3,5}, but also as somites or segmental muscle blocks^{6,7}. The polybranchiaspids did not acquire their bony armour until they achieved their definitive size, hence it is necessary to postulate the existence, in the young stages, of serially arranged somites on either side of the central nervous system along the dorsal part of the head and trunk region.

These structures can be interpreted as segmental muscle blocks above the gill pouches, contributing to the pumping mechanism. The gills were unlikely to have been in direct contact with the dorsal armour; there would have been some somitic musculature intervening.

The preservation of the internal anatomy of the polybranchiaspids makes possible direct comparisons with living agnathans, the hagfish⁸ and lampreys^{9,10}, as well as fossil, cephalaspids and heterostracans^{6,7,11-14}. The living forms are characterised by their eel-like shape and absence of a bony external skeleton, scales or paired fins, all of which appear to have been secondarily lost. Several further features unite the two groups: a single external nasohypophyseal opening, paired olfactory lobes, pore-like gill openings, horny teeth on the tongue and the haemoglobin molecule made up of a single polypeptide chain (in contrast to the four of all higher vertebrates). Many of the differences are connected with their different modes of life. The hagfish is highly specialised in its reduction of sense organs, the eyes are reduced to the merest vestiges, there is no pineal organ and only a single canal in the ear, generally considered homologous to the posterior semi-circular canal and a combined utricle-sacculus element. The only feature that can be directly compared with that of the polybranchiaspids is the nasohypophyseal opening, which is terminal. The venous system of the hagfish, with its ventral cardinal 'heart' deep to the anterior cardinal sinus, is not comparable to the lateral head vein and occipital sinus of polybranchiaspids. The well developed eyes and ear regions and the configuration of the brain of the polybranchiaspids render hagfish affinities unlikely.

When the polybranchiaspids are compared with the lamprey, the similarities are striking: there is a dorsal median opening with a pineal organ behind. There are two semi-circular canals of the ear and the configuration of the brain is similar. The gill pouches each had separate openings to the exterior as in lampreys. In contrast, however, the polybranchiaspids appear to have paired nasal sacs and the pineal was covered by the bony armour. The details of the internal anatomy strongly indicate a close relationship to lampreys. The cephalaspids possessed the same basic similarities and differences with the polybranchiaspids as the lampreys, but show further similarities in the degree of endocranial ossification and in the venous system, which is comparable to that of the primitive cephalaspid *Tremataspis*. The polybranchiaspids were closely related to the cephalaspids, but can be excluded from them on account of their lack of dorsal and lateral sensory fields, the corresponding lobes of the hindbrain, the large dorsal median opening, covered pineal and paired nasal capsules.

Finally, the polybranchiaspids can be compared with the heterostracans, with which they have been generally associated. They are similar in possessing double nasal sacs and a covered pineal but differ in the endocranial ossification and separate gill openings. The recognition that the dorsal median opening was not a mouth, excludes the polybranchiaspids from the heterostracans⁴.

The details of the internal anatomy confirm that the polybranchiaspids belong to an independent group, the Galeaspida, allied to lampreys and cephalaspids within the Cephalaspidomorphi division of the Agnatha.

Specimens were drawn in Peking by Jennifer Halstead.

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Dioecious *Solanum* species of hermaphroditic origin is an example of a broad convergence

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Unlike the taxa in some genera which show a range of variability in floral morphology, species of *Solanum* deviate very little from the typical hermaphroditic flower. The known deviant taxa fall into two classes: most are andromonoecious (male and hermaphroditic flowers on the same plant), and the remaining few are androdioecious (male flowers on one plant, hermaphroditic flowers on another)¹. I report here evidence for the first instance of a truly dioecious species of *Solanum*, a significant discovery in the light of the floral uniformity of this large (second only to *Senecio*) and economically important genus. The most closely related species and species groups comprise only hermaphroditic forms; this dioecious species is among the few non-hermaphroditic forms native to the New World, the area of greatest species diversity of the genus. Furthermore, as shown below, the species is characterised by a functionally, though not morphologically, dioecious condition. Finally, this demonstration that the two floral forms represent different sexes of the same species, rather than (as originally described) different species, impels closer analysis of other species pairs.

The plants studied were originally collected in mountainous regions of the states of Mexico and Michoacan in Mexico, and San Marcos in Guatemala. The specimens collected were identified using Correll's monograph² as *Solanum appendiculatum* and *Solanum inscendens*. These species are non-tuberous (although relatively closely related to the potato) woody perennials that grow to a length of 3-10 m up tree trunks and rock surfaces. The primary character used to separate these two species in keys is the length of the style in relation to the staminal column (short style, *S. appendiculatum*; long style, *S. inscendens*). When the initial collections were grown in greenhouses, seeds from each fruit collected produced both long style and short style plants. Subsequently, seed populations from 24 collections have been grown to generate the sex ratio given in Table 1 line (1). It is clear from ratios derived from populations of seeds collected in the field, and from a small artificially derived F₁ population (line 2), that the primary sex ratio is about two long (L-functional female, see below) to one short (S-functional male). However, the sex ratio derived from randomly selected populations in the field is nearer to 1:1. The latter ratio is closer to that usually reported for dioecious taxa³⁻⁵. At present, too little is known of the field biology of this *Solanum* taxon to speculate on which factors alter the primary sex ratio.

The relationship between the long- and short-style plants was assayed with crossing studies and with observations of pollen tube growth in styles. The results of these two analyses provide strong evidence that this species is functionally dioecious. More than 400 crosses were attempted among the four possible combinations of short- and long-styled flowers. The only successful combination involved L♀ and S♂ (Table 2). Seeds resulting from this combination were viable, and the mature F₁ offspring were fully fertile. Studies of pollen tube growth⁶ add

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Table 1 Features of the sex forms

Features	Long-style form L	Short-style form S
(1) Sex ratio (greenhouse)	2.02 (107)	1.0 (53)
(2) Sex ratio in a greenhouse F ₁ population	1.75 (7)	1.0 (4)
(3) Sex ratio (field collections)	1.0 (23)	1.22 (28)
(4) Flowers per inflorescence	34.0 (30)	34.27 (30)
(5) Pollen grains per flower	82,750 (6)	129,465 (5)
(6) Pollen stainability	78.6% (119)	79.4% (77)
(7) % Pollen grains: inaperturate tricolporate	100% 0% (2,281 grains, 20 collections)	0% 100% (2,587 grains, 25 collections)
(8) Ovules per ovary	11.26 (42)	11.28 (36)

The upper of the two sets of numbers represent averages, while the lower sets, in parentheses, represent sample sizes.

further confirmation that the $L\varnothing \times S\delta$ combination is the only one functional. With a single exception, pollen tubes were produced only when pollen from short-style flowers was placed on stigmas of long-style flowers (Table 2). Virtually all other combinations, including attempts at self-pollination (columns *E* and *F*, Table 2), failed to yield pollen tubes. These data are also interesting in relation to the question of potential mechanisms responsible for crossing success. Perhaps crosses fail and no pollen tubes are produced because of chemical interactions between stigma and pollen, such as those involved in typical *S*-allele (incompatibility) systems⁷. Or perhaps, as implied below, the pollen from the long-style flowers is physically unable to germinate.

Analysis of field collections and greenhouse-grown plants indicates that both long- and short-style forms possess an equal number of flowers per inflorescence (Table 1, line 4). This 1:1 ratio for inflorescence size is incongruent with the usually larger inflorescences of males reported by Opler and Bawa⁴ for dioecious tropical trees. There is, of course, no reason to expect that every species will follow the general pattern Opler and Bawa have described. Furthermore, because *Solanum* flowers reward potential pollinators with pollen rather than nectar¹, one could expect the female inflorescences to remain as large as the male and thus not suffer a reduced frequency of visits because of insufficient reward.

Although the sex ratio is not male biased and inflorescence sizes are the same, counts⁸ of the number of pollen grains per flower (Table 1, line 5) in the two sex forms are significantly different. The short-style flowers bear greater than 55% more pollen grains. Perhaps the smaller quantity of pollen grains in the female flowers (though not so small as to reduce the frequency of visits by pollinators) is one manifestation of the selection for unisexuality. Although the quantity of pollen is less in the long-styled flowers, two tests of viability (aniline blue in lactophenol, acetocarmine) showed that both flower types produced equally stainable pollen (Table 1, line 6). However, such tests can only estimate viability and not whether pollen is actually functional. The data given here for crossing and pollen tube growth are one indication that the pollen from long-style flowers is not functional. Light and scanning electron microscope studies of acetolysed pollen give another. The long-style flowers always bear inaperturate grains and the short-style flowers the more typical tricolporate types⁹. The lack of development of colpae and pores in the pollen of the long-style form may be one of a suite of adaptations to functional femaleness. Inaperturate pollen, presumably quite functional, is

reported from several families including the Solanaceae¹⁰. However, the data reported here seem to be a strong indication that while pollen from the long-style flowers may be a suitable reward for floral visitors, it is not otherwise involved in the reproductive process.

Except for the difference in style lengths, the gynoecium (stigma, style and ovary) shows few morphological modifications. Contrary to expectations, the short-style flowers bear as many ovules as the long-style flowers (Table 1, line 8). This seems a strong indication that the dioecy in this species is still in an early stage of evolution.

The discovery of a new breeding system in a genus as large and invariable for this feature as *Solanum* is unusual. The important point is that the long- and short-style flower forms do not represent two species which hybridise. Crosses in the right combinations can be made with ease, and the resultant F₁ plants are fully fertile. In addition, greenhouse populations consistently give a 2L(♀):1S(♂) segregation ratio. Furthermore, the data for crossing and pollen tube growth show clearly that this is not a case of heterostyly. This is in agreement with previous conclusions that true heterostyly is unknown in the Solanaceae¹¹. Rather, the long- and short-style flowers are simply breeding morphs of a single dioecious taxon.

What evolutionary advantage the dioecious system confers on this taxon is perplexing. The usual explanation that dioecy arises in a self-compatible taxon as a way of ensuring outcrossing^{3,12} is certainly possible. However, it is somewhat less likely here because most closely related species tested possess a well developed system enforcing obligate outcrossing^{7,13}. Bawa and Opler³ have argued that dioecy may be selected in hermaphroditic flowers in preference to outbreeding mechanisms such as *S*-alleles to enhance effective pollination. They argue that if the number of flowers on an individual is high, most pollination will involve transfer of ineffective self pollen. Such pollen could cover the stigmatic surface so that pollen brought from another plant may, although capable of it, be unable to effect pollination because stigmatic space is unavailable. This argument was proposed for tropical trees with a very large number of flowers. It may apply to the dioecious *Solanum* described here because it has larger inflorescences with more flowers open at any one time than most closely related species^{2,13}. However, the fact that females bear anthers with pollen (although significantly less pollen) lessens the force of this argument for this species.

The dioecy described here is also of particular interest because there is no precedent for unusual sex forms in any of the closely related species groups¹. Several authors^{14,15} have argued that dioecious taxa frequently evolve from gynodioecious or less frequently androdioecious ancestors. Gynodioecious taxa are unknown in *Solanum*, and although androdioecious taxa have been reported, they are, like most species with unusual sex forms in the genus, native to the Old World and are placed in a different subgenus¹. Thus the development of dioecy described here does not represent the culmination of a linear evolutionary

Table 2 Results from cross pollination and pollen tube studies

	Combinations					
	A L♀ × S♂	B S♀ × L♂	C L♀ × L♂*	D S♀ × S♂*	E S self†	F L self†
Crosses attempted	102	101	102	102	0	0
Crosses successful	64	0	0	0		
% successful	63%	0%	0%	0%		
Styles: with						
pollen tubes	44	0	0	1	0	0
without						
pollen tubes	0	23	20	22	5	6

* Crosses between flowers of different plants with same style lengths.

† Crosses between flowers on the same plant.

series, but rather a broad convergence on a set of apparently advantageous breeding systems. Furthermore, Crowe¹⁶ has suggested that most dioecious species evolved from monoecious (primarily) or heterostylous taxa; the lack of either of these conditions in *Solanum* precludes their application to this case. The fact that the separation of sexes is still only functional, and not morphological, very likely indicates a system in the early stages of evolutionary development. This situation provides a special opportunity which, as Crowe proposed¹⁶, may lead to a better understanding of the origin and evolution of incompatibility mechanisms among the angiosperms in general.

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Length and evolutionary stability of food chains

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Grazing food chains in natural ecosystems tend to be relatively short, usually involving no more than four or five trophic levels^{1–3}. Cohen⁴ describes 19 food webs with a total of 102 top predators. Recent independent analyses of these data by Cohen⁴ and Pimm (see ref. 5) show that most top predators occupy the third trophic level. However, as Slobodkin³ has observed, "It is possible to conceive of predators that are small enough and ferocious enough to attempt to live off the top of a trophic pyramid, and thereby become a seventh, or even an eighth, trophic level. It is by no means clear why this situation has not occurred." Our answer, which we discuss here, incorporates energy transfer calculations introduced by Hutchinson¹ and Odum and Odum² within the framework of the evolutionarily stable strategy (ESS) developed by Maynard-Smith and Price^{6,7}. We argue that food chains should collapse to the shortest possible length compatible with biochemical and physiological constraints. This minimal length is frequently three, and is largely independent of primary productivity.

Odum and Odum² consider the inefficiency of energy transfer. If each species can transmit only a fraction (usually between 5% and 20%) of its incoming energy to the next level, this sharply limits the length of food chains. Although any such limit scales as the logarithm of primary productivity, Pimm and Lawton⁸ observe that 'food chains are not noticeably shorter in barren Arctic and Antarctic terrestrial ecosystems compared with a productive tropical savannah or the fish guilds of a tropical coral reef,' although primary productivity varies by a factor of 10^4 . Hutchinson² proposes that pressure to increase predator efficiency could even shorten food chains. An efficient higher predator, with limited food supply, might eliminate his prey and be forced either to drop to a lower trophic level or to become extinct. Pimm and Lawton⁸ propose a third explanation involving Lotka-Volterra dynamics (see refs 5, 9 for reviews). Our explanation considers the evolutionary stability of alternative trophic strategies. Trophic levels are defined by assigning plants the first trophic level, herbivores the second and primary carnivores the third. More generally, a species which feeds primarily on the n th trophic level is assigned the $(n+1)$ st trophic level. The classical energy transfer argument states that lower trophic levels offer a richer thermodynamic environment (in terms of free energy) than do higher trophic levels. Following the analysis of optimal foraging and resource partitioning of MacArthur and Pianka¹⁰, a predator's strategy may be described as optimal if the predator exploits the thermodynamically richest accessible environment. We shall argue that such optimal strategies are evolutionarily stable, whereas alternatives are not.

Consider a fourth-level species, one which feeds primarily on carnivores. Its nutritional needs could be satisfied at a lower trophic level by herbivores, as carnivores and herbivores are nutritionally interchangeable. In the absence of additional non-nutritive constraints, members of this species could always drop to a lower trophic level and compete with their former prey for the thermodynamically richer herbivore environment. As long as the marginal energy gain from feeding on herbivores is greater than feeding on carnivores, the ESS will be to concentrate on herbivores, but to feed on carnivores encountered in defence or competition. This assumption on energy gains is justified by the usual range of transfer efficiencies, and the probable greater unit costs of successfully attacking carnivores.

More precisely, let $h(t)$ be the energy gain from expending an effort t on feeding on herbivores. Similarly, let $c(t)$ be the gain from an effort t on carnivores. We assume that both $h(t)$ and $c(t)$

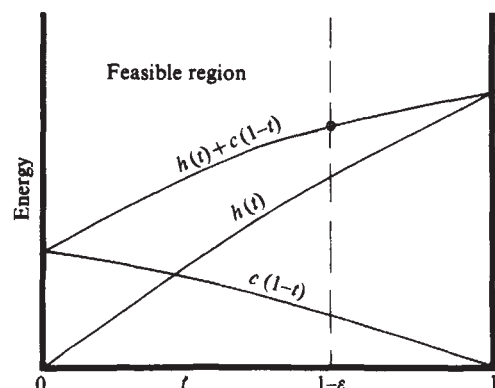


Fig. 1 The ESS of maximum feasible energy gain. The graph shows the net energy gain $h(t) + c(1-t)$ from a strategy of spending effort t on herbivores and $1-t$ on carnivores, its components $h(t)$ and $c(1-t)$ from herbivores and carnivores, and the required energy ϵ expended on feeding on carnivores. Thus, $0 \leq t \leq 1-\epsilon$. The maximum energy gain occurs at $t = 1-\epsilon$ under our assumptions.

are increasing functions, convex because of 'diminishing returns', and close to linear, and that $c(t) \ll h(t)$ —the ratio $c(t)/h(t)$ represents an effective transfer efficiency. Now, consider a strategy of expending an effort t on feeding on herbivores and $1-t$ on feeding on carnivores. Here $0 \leq t \leq 1$. We seek to maximise the total energy gain $h(t) + c(1-t)$ subject to the constraint that the organism expend at least a small effort ε in competing with (and incidentally feeding on) potential carnivore prey. Many factors influence ε , including territorial behaviour, ability to flee and population density. As long as

$$\left. \frac{dh(\tau)}{d\tau} \right|_{\tau=1-\varepsilon} > \left. \frac{dc(\tau)}{d\tau} \right|_{\tau=1-\varepsilon}$$

or simply, $h'(t) > c'(1-t)$, for $0 \leq t \leq 1-\varepsilon$, this maximum will occur at $t = 1-\varepsilon$ (Fig. 1). Therefore, from the above assumptions, if relatively little energy ε need be expended for competition, such an organism should adopt the ESS of concentrating (spending effort $1-\varepsilon$) on herbivores.

The option of switching to a lower trophic level is not available to first-level carnivores because of nutritional differences between plants and herbivores; herbivores clearly face far stricter constraints. Furthermore, these nutritional requirements arise from synthetic and degradative specialisations which are selectively advantageous. However, a parasite may be subject to different dynamics, and not always select the most numerous host because its host constitutes its entire thermodynamic and spatial environment, and is not a potential competitor under alternative trophic strategies. Thus, on the spatial scale of an individual parasite^{6,7}, a herbivore host might offer no advantages over a carnivore. It is also conceivable, but energetically unlikely, that diminishing returns could make $h'(t_*) = c'(1-t_*)$ for some $t_* < 1-\varepsilon$. This yields a mixed strategy^{6,7}, with effort t_* expended on herbivores and $1-t_*$ on carnivores. Finally, the case of concave $h(t)$ or $c(t)$ is also energetically unlikely.

We have shown that in the absence of non-nutritive constraints, grazing food chains should evolve towards the evolutionarily stable length of three. Parasitism and opportunistic feeding allow for some chain lengthening. Longer chains may also exist transiently, possibly being relatively long-lived, or may persist due to special constraints such as size and habitat compatibility of predator and prey. Differences in nutritional value of predators and prey are unlikely to be as significant for protists as for metazoans, but size constraints may be more important. These factors are independent of primary productivity. Further, our argument that optimal foraging subject to biochemical and physiological constraints is an ESS applies to all trophic levels and strategies, independently of the detailed structure of the food web. We therefore predict that the length of food chains will be relatively constant over wide ranges in primary productivity. This agrees well with field data^{4,5}.

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Male lions in large coalitions gain reproductive advantages

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Cooperation between two or more individuals has been shown to yield short-term benefits in several vertebrate species¹⁻⁴, and various hypotheses have been developed to explain the evolution of cooperative behaviour⁵⁻⁷. However, until now there has been no evidence to show that such cooperation actually does confer lifetime's reproductive advantages on more than one member of the coalitions concerned^{8,9}. Long-term studies of wild lions (*Panthera leo* L.) have now provided such evidence. We show that, compared with singletons and pairs, male lions in groups of three or more can more reliably gain tenure of female prides, retain tenure for longer, mate with more different females, and produce more surviving offspring; thus each individual has higher fitness through cooperation.

Lions were observed in the Serengeti National Park and in the adjacent Ngorongoro Conservation Area, Tanzania, in 1966-69 by G. B. Schaller²; in 1969-73 by B.C.R.B.^{10,11}; and in 1974-78 by J.D.B. and J.P.H.¹². Observations on the same prides in comparable habitats were spread over periods of 3-12 years. All members of these prides were known individually by natural markings¹³.

A lion pride is a matrilineal group of 2-18 (mean 6)^{2,12,14} genetically related females, plus their cubs. Young male lions usually leave their natal pride as a group of relatives shortly before sexual maturity. Then, as a coalition, they gain and retain sole possession of a pride for a variable period, excluding rival male groups. The male group's tenure, and therefore its effective reproductive life¹⁰, typically ends when they are displaced from their pride and their area by a new group of males. Here we compare aspects of the reproductive success of the male groups of different sizes.

Table 1 The frequency of male lion groups of different sizes, and the probability that they will have tenure of a pride of lionesses

No. of males in group	No. of male groups		Probability of having tenure	Significance of difference
	Total	No. which had tenure		
1	23	4	17.4%	<0.01
2	39	23	59.0%	
3	12	11	91.7%	
4	4	4	100.0%	
5	1	1	100.0%	
6	3	3	100.0%	
7	1	1	100.0%	

Total 183 adult males (that is, ≥ 4 yrs old), observed in the Serengeti region over more than 2 yrs during 1969-78.

Table 1 shows that single males in the Serengeti region were rarely successful in gaining tenure of a pride (4/23), whereas pairs quite often were (23/39 pairs); large groups (3 or more males) were almost always in possession of a pride (20/21 groups).

Having gained tenure, large male groups were also more successful in retaining it. Data henceforth refer to 25 male

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groups. Figure 1a shows that compared with pairs (mean tenure 18 months), groups of 3 males stayed at least 2.3 times as long, and groups of six males stayed over 3.8 times as long with their prides. Note that the tenure periods of all the large groups (but of only some of the small groups) are underestimated because they extended beyond the beginning or end of the observation periods; thus the differences shown are conservative.

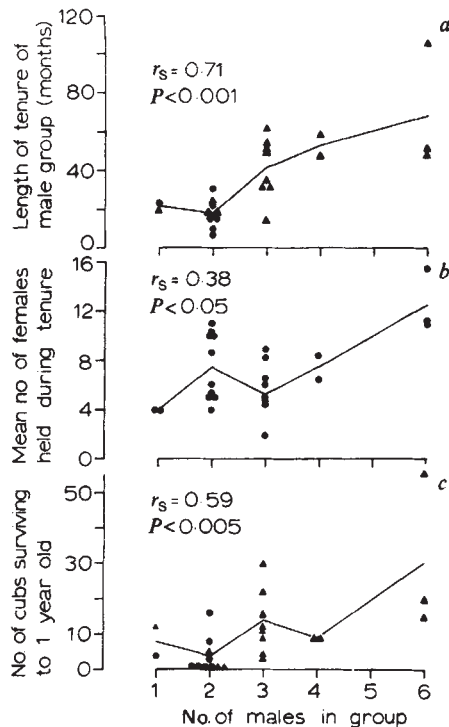


Fig. 1 Aspects of the reproductive success of male lion groups of different sizes. *a*, The numbers of months that each group retained tenure of a pride or prides in the study area. *b*, The mean numbers of females concurrently in the possession of the male groups, averaged over their observed period of tenure. *c*, The total numbers of cubs fathered during the male groups' tenure which survived to 1 yr old (by which age 77% of cub mortality has occurred¹⁰). ▲, Points which are minimum figures because the male groups' periods of tenure extended beyond the beginning or end of the study period; ●, points which are not underestimates.

Large male groups, especially after staying with one pride for about 2 years, may gain access to one or more neighbouring prides as well. Their concurrent tenure of several prides gives them access to more oestrous females. Figure 1b shows that in general only the very largest male groups (>4) held appreciably more females concurrently. They did so not by holding larger prides but by holding two or more at the same time.

When a lioness comes into oestrus (lasting 2–5 d), any male in the group consorts alone with her, mating repeatedly. Having companions should subdivide a male's share of consortships; however, the available data show no significant negative correlation between the number of males in a group and their consortship scores ($n = 50$, $r_s = +0.14$, $P > 0.2$). Consortship scores were calculated as a percentage of total sightings, excluding males sighted fewer than 10 times. For 50 males, $\bar{x} = 19.6\%$, $s.d. = 8.6$).

There is no detectable hierarchy within male groups^{2,14}, all partners sharing their mating duties with remarkable equity. For example, no male was seen consorting more than 2.7 times as often as his partner(s), and in the most often observed group of 6 males ($\bar{x} = 81$ sightings of each male) no male was involved in >22% or <9% of all consortships seen. Overall, the difference

between consortship scores was smaller and far less variable between members of the same coalition ($\bar{x} = 7.6$, $s.d. = 5.6$, $n = 62$) than between members of different coalitions ($\bar{x} = 11.0$, $s.d. = 26$, $n = 1,138$; F test $P < 0.001$).

A female may sometimes change consorts during her oestrous period, and thus mate with more than one male. There are no indications that particular males are likely to be in consort with her at particular times during her oestrus. Copulation may occur as frequently as every 15 min, so only a minute proportion of copulations (<1%) leads to the birth of cubs^{2,10}. Hence, unlike some species^{15,16}, for lions the number of copulations would not provide a reliable indicator even of short-term reproductive success. Nor would the number of litters born, because cub mortality is high and because high mortality of lion cubs raises the birth rate¹⁰.

The best measure of lifetime reproductive success is the number of surviving offspring produced. Figure 1c shows that the total number of cubs surviving to 1 year old is correlated with their fathers' group size ($r_s = 0.59$, $P < 0.005$). The effect is achieved mainly by staying for longer and so fathering more cubs, rather than by improving their probability of survival. Nonetheless, some cub mortality is associated with male takeovers^{10,14}, caused by the new males killing or excluding some of their predecessors' offspring. Long tenure defers and so reduces this mortality.

We have shown above that larger groups as a whole do better. Figure 2 shows that, assuming equally shared paternity, each individual male in a large group achieves greater expected lifetime's reproductive success (that is, has greater fitness) than he would have achieved in a small group. Comparing the fitness

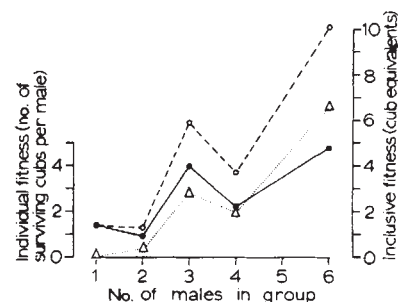


Fig. 2 Individual fitness and inclusive fitness of individual male lions in groups of different sizes. The line for mean individual fitness (●) is derived from Fig. 1c assuming equally shared paternity of the cubs born during the males' tenure, and multiplying by the different probabilities of achieving tenure at all (from Table 1). The line for a male's mean inclusive fitness (○) is the sum of first, the number of cubs he has fathered (that is, his mean individual fitness) and second, the number of cubs fathered by his companions multiplied by 0.22 (that is, devalued by their coefficient of relatedness to him). The lowest line (Δ) shows the inclusive fitness of a male who fathers no cubs of his own.

of males in large groups of 3 or more ($n_1 = 13$ groups) with that of males on their own or in pairs ($n_2 = 12$), the 3.8-fold difference is highly significant ($P = 0.001$, Mann-Whitney U -tests). The advantage does not continue to increase: thus the males in the few (5) very large groups (≥ 4 males) do not have higher fitness than the males in groups of 3.

The male lions in a coalition are generally relatives: it has been calculated¹⁴ that a male's average degree of relatedness to his companions is 0.22, and that this is almost independent of group size. Cubs fathered by other males in the group also contribute to his inclusive fitness^{5,17}. We ignore for present purposes the common increment to inclusive fitness produced by the reproduction of all other relatives everywhere, and consider only the contribution by other members of the male group. It can

generally be assumed that a male forms a coalition with similar-age relatives if he has them. (This was true of 19 out of 21 coalitions whose origins were known; the other two cases were a pair of strangers and a trio of two brothers and a stranger.) The inclusive fitness of each male can thus be calculated, and is illustrated in Fig. 2. There is a marked increase in the inclusive fitness of a male the more related companions he has, such that the inclusive fitness of a male in a large group is 5.7 times that of a lone male or of one of a pair. The initial individual advantage gained through cooperation is amplified through kin selection^{5,14,17}.

We have assumed in our calculations that paternity (like consortships) is shared equally by all members of a coalition; naturally this is not certain, nor can it be tested in wild populations. However, it can be shown that even if paternity were unequally shared, the inclusive fitness of every member of a large kin-related coalition would still exceed that of a lone male or one of a pair. In the most extreme and improbable case, although a male might sire no cubs of his own, his support of the coalition would enhance the survival of his relatives' cubs. Other things being equal, this would therefore be a better genetic strategy for him than leaving the coalition (Fig. 2).

If members of large groups generally enjoy higher reproductive success, why do not all male lions form large coalitions? It is apparently difficult to form a successful coalition, probably because the bonds necessary have to be developed early in life. Although single unrelated males can sometimes form stable pairs eventually, they seem unable to form or join larger groups, which are intolerant towards strangers. Thus the size of male groups is determined by lion prides' ability to produce and rear male offspring. The small litter size (mean 2.5 cubs¹⁰), overall even sex ratio², and high (~75%) cub mortality^{2,10,12} mean that a large male peer group can reliably result only if a considerable number of lionesses in a large pride give birth synchronously. There are indications that prides in fact manage to produce large groups of young males more often than expected (J.D.B. and J.P.H., unpublished data), and how they do so is being investigated. Why they do so is now clear: females who manage to produce large groups of male cubs produce through them disproportionately many grandcubs. Previous cooperation by their mothers (in synchronising births¹⁰ and in rearing cubs cooperatively^{2,10}) is a prerequisite for the high lifetime's reproductive success of male lions cooperating in large coalitions.

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Inhibition of mixed lymphocyte response by monoclonal antibody specific for a rat T lymphocyte subset

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Cell-surface differentiation antigens which are associated with particular lymphocyte subsets are likely to be molecules which mediate specific functions of the cells which display them. One way to investigate this hypothesis is to see whether antibodies directed against such antigens will inhibit functional systems. This approach can be attempted with confidence if monoclonal antibodies are used, as these react with one antigenic determinant only and can be used in purified form. In this report we show that a monoclonal antibody which is specific for a subset of rat T lymphocytes inhibits the mixed lymphocyte response (MLR). The antibody acts on the responder cells without killing them.

The W3/25 mouse monoclonal antibody is specific for thymocytes and a subset of T lymphocytes in the rat and the cells in thoracic duct lymph that express the target antigen mediate helper and graft-versus-host functions^{1,2}. The W3/25 antigen is relatively sparsely represented on the cell membrane as only about 15,000 molecules of W3/25 antibody are bound at saturation to thymocytes or T cells, yet more than 5×10^5 anti-Thy-1.1 antibodies bind per rat thymocyte¹.

The effect of W3/25 IgG on the MLR is shown in Fig. 1. In these experiments 2×10^5 lymph node cells from PVG/c (RT1^c) rats were used as responders to 5×10^5 irradiated spleen cells from the congenic strain HO.B2 (RT1^b) in 0.2 ml cultures as described by Antczak *et al.*³. The W3/25 antibody was added to the cells at the start of the culture period and 0.5 μ Ci of tritiated thymidine (³H-TdR) were added after 72, 96 or 120 h in culture. After a further 18 h the cells were assayed for incorporated label. All assays were carried out in triplicate. Concentrations of W3/25 IgG up to 2.5 ng ml⁻¹ in 0.2 ml gave no inhibition but at a concentration of 25 ng ml⁻¹ the antibody reduced incorporations of ³H-TdR to a plateau level which was not affected by further increases in antibody concentration. We have observed this inhibition without exception in nine experiments where W3/25 antibody was added to MLRs. In 21 independent observations spread throughout these experiments the mean incorporation of fully inhibited cultures compared with control MLRs was 18.2%, standard deviation 7.3%. Within any one experiment the inhibition became greater, relative to a control MLR, with time up to 120 h. The inhibition was not confined to MLRs with PVG/c versus HO.B2 cells but was also seen in the reverse combination (see also Table 2) and in two other strain combinations which were tested (AO versus DA and PVG/c versus DA).

Other monoclonal antibodies were tested, including W3/13 antibody, which labels all T cells but not B cells¹; MRC OX 1 antibody, which labels all leukocytes⁴ and a monoclonal mouse IgG anti-Thy-1.1 antibody called MRC OX 7 (W. R. McMaster, unpublished) which labels thymocytes but only a small proportion of peripheral T cells in the rat⁵. These antibodies all bind at levels per labelled cell which are at least 2.5 times greater than W3/25 antibody, yet none inhibited the MLR, and W3/13 antibody gave a consistent stimulation of ³H-TdR incorporation (data for W3/25 and W3/13 in Fig. 1; that for MRC OX 1 and MRC OX 7 not shown). For W3/25 and W3/13 antibodies, the extent of binding was checked at 37 °C and greater than 90% saturation was achieved with the same assay conditions used for the MLR at 25 ng ml⁻¹ for W3/25 and 5 μ g ml⁻¹ for W3/13 antibody (D. W. M., unpublished). Furthermore, W3/13 antibody failed to have an inhibitory effect even at a concentration

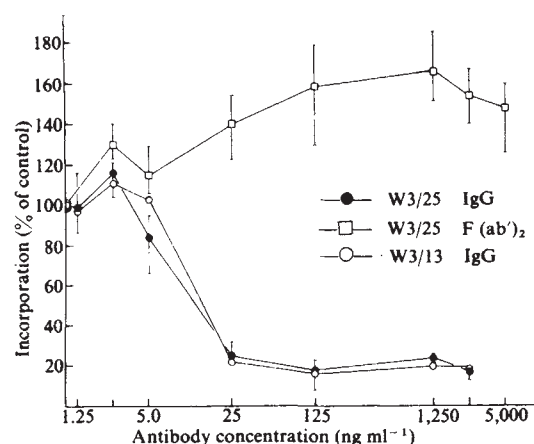


Fig. 1 Effect of W3/25 and W3/13 antibody on the MLR. Data from two independent experiments have been normalised by putting the control MLR incorporation at 100%. The control values were 22×10^3 c.p.m. for the W3/25 experiment and 40×10^3 for the W3/13 experiment. The W3/25 and W3/13 antibodies were added as pure IgG or $F(ab')_2$ prepared by standard procedures. The $F(ab')_2$ contained no IgG detectable by SDS-polyacrylamide gel electrophoresis. The W3/25 IgG contained no myeloma kappa chain since the W3/25 HL hybridoma was used². W3/13 contained some myeloma kappa at a minor level compared with the antibody L chain. Ranges are shown for W3/13 IgG and W3/25 IgG. Ranges for W3/25 $F(ab')_2$ were generally similar to those for W3/25 IgG.

of $50 \mu\text{g ml}^{-1}$ (data not shown). These observations mean that it is very unlikely that W3/13 antibody did not have an inhibitory effect because it was not used at an adequate concentration.

$F(ab')_2$ fragments prepared from W3/25 IgG also inhibited the MLR and exhibited the same dose-response curve as purified IgG (Fig. 1), indicating that the Fc portion of the antibody molecule is not involved. The serological specificity of the effect was shown by the fact that the inhibition was relieved by absorption of the antibody with rat thymocytes, whereas absorption with rabbit thymocytes had no effect (Table 1).

The effect of W3/25 antibody on responses to T-cell mitogens was investigated by incubating lymph node cells with concanavalin A (Con A) and phytohaemagglutinin (PHA) in the presence of antibody. Cell proliferation caused by these agents was assayed by ^3H -TdR incorporation after 48 h in culture and was not significantly inhibited by W3/25 antibody at a concentration as high as $2.5 \mu\text{g ml}^{-1}$ (Table 1). This is 100 times the level required to give maximal inhibition of the MLR. The potent inhibition of cell proliferation exerted by W3/25 anti-

Table 1 Effect of various treatments on the MLR of PVG/c lymph node cells responding to irradiated HO.B₂ spleen cells

Treatment	^3H -TdR incorporation (c.p.m. $\times 10^{-3}$ per 18-h pulse)
<i>Rat absorption</i>	
Control MLR (2×10^5 cells)	54
+unabsorbed W3/25 antibody	11
+W3/25 absorbed with 6×10^7 rat thymocytes	72
<i>Rabbit absorption</i>	
Control MLR (2×10^5 cells)	52
+unabsorbed W3/25 antibody	11
+W3/25 absorbed with 5×10^7 rabbit thymocytes	9
<i>PHA stimulation</i>	
Control (3.5×10^4 cells)	35
+25 ng per ml W3/25 IgG	37
+2,500 ng per ml W3/25 IgG	32
<i>Con A stimulation</i>	
Control (3.5×10^4 cells)	32
+25 ng per ml W3/25 IgG	58
+2,500 ng per ml W3/25 IgG	31
Control MLR with no preincubation	0 h 38
2×10^5 cells cultured for various times without addition of antibody before MLR	4 h 61
	8 h 73
	24 h 37
	48 h 15
2×10^5 cells cultured for various times with W3/25 IgG (25 ng ml^{-1}) before MLR	4 h 40
	8 h 24
	24 h 63
	48 h 12

In the absorption experiments W3/25 antibody was used in the form of spent tissue culture medium from cultures of cells secreting W3/25 antibody ($0.4 \mu\text{l}$ per culture) while in the other experiments pure W3/25 HL IgG was used. Con A was used at a final concentration of $10 \mu\text{g ml}^{-1}$ and PHA at $20 \mu\text{g ml}^{-1}$.

body when cells are stimulated by alloantigens does not, therefore, extend to the proliferation induced by T-cell mitogens.

To discount the possibility that the responding cells in the MLR are killed by the antibody, lymph node cells were cultured without stimulator cells at 37°C in the presence or absence of W3/25 antibody (25 ng ml^{-1}). They were then washed twice and resuspended in medium lacking antibody and assayed in a standard MLR. These experiments showed that cells could be incubated in the presence of antibody without any reproducible indication that this affected their ability to respond to

Table 2 Alloreactivity of W3/25⁺ and W3/25⁻ lymphoid populations

				^3H -TdR incorporation (c.p.m. $\times 10^{-3}$ per 18-h pulse)		
Incubation period	Responders	Stimulators	Unstimulated control	MLR	MLR + W3/25 IgG (25 ng ml^{-1})	
Expt 1	72 h	2×10^5 W3/25 ⁺ (PVG/c)	5×10^5 HO.B2 spleen cells	0.1	57	—
	72 h	2×10^5 W3/25 ⁻ (PVG/c)	5×10^5 HO.B2 spleen cells	0.1	7	—
Expt 2	72 h	2×10^5 W3/25 ⁺ (PVG/c)	5×10^5 HO.B2 spleen cells	1	17	—
	72 h	2×10^5 W3/25 (PVG/c)	5×10^5 HO.B2 spleen cells	0.2	4	—
Expt 3	96 h	2×10^5 HO.B2 lymph node cells (unfractionated)	5×10^5 irradiated W3/25 ⁻ PVG/c thoracic duct lymphocytes	2	18	3

Each value is the mean of triplicate assays where the range was always $\pm 15\%$ of the mean. In experiments 1 and 2 MLR's were also done with responders at 1×10^5 and 5×10^5 cells per culture. Typical dose-response curves were obtained and the ratio of W3/25⁺:W3/25⁻ responses was similar to that obtained with 2×10^5 responders.

subsequent alloantigenic stimulation. The results of one such experiment are shown in Table 1 with preincubations for various times. At only one time point (8 h) did there appear to be any effect on the subsequent MLR. In another experiment (data not shown) where cells were preincubated for 24 or 48 h no inhibition was observed.

These findings show that W3/25 antibody inhibits the MLR quite specifically without killing the cells. This effect prompts questions about the role of W3/25 positive cells in the MLR. It might be expected that the major part of the cell proliferation in the MLR is due to W3/25 positive cells and that in conditions of inhibition by W3/25 antibody these cells have no role as responders. This possibility was investigated using a fluorescence activated cell sorter to prepare populations of W3/25-positive and -negative cells from thoracic duct lymph². In two independent experiments, covering a range of responding cell doses, and culture periods of 72 and 96 h, the incorporation of ³H-TdR by W3/25-positive cells was found to be at least four times that of the W3/25-negative cells when equal numbers of responding cells were compared (Table 2).

In a third experiment, stimulator cells were depleted of W3/25-positive T cells in the cell sorter to a level of 0.8% contamination and used to stimulate fresh lymph node cells. The W3/25-negative cells acted efficiently as stimulators (Table 2) and the resultant MLR could be inhibited by W3/25 antibody

giving the same dose-response curve as that shown in Fig. 1 (incorporation by culture treated with 25 ng ml⁻¹ W3/25 IgG = 19.3% of control MLR). Similar results were obtained in another experiment. It might be argued that the stimulator effect in these experiments was dependent on very small numbers of W3/25-positive contaminating cells. However, this is unlikely as although W3/25-positive cells were able to stimulate an MLR, they were no more potent than W3/25-negative cells and when added at 2×10^4 cells per culture gave no detectable stimulation of the MLR (M. W., unpublished). This number of W3/25-positive cells is five times greater than the estimated number of contaminating cells present in the W3/25-negative stimulators used in experiment 3 of Table 2.

These results suggest that the targets of the antibody are the responding cells rather than the stimulating cells, and the inhibition data indicate a blocking function for added W3/25 antibody. It is thus possible that the W3/25 molecule is of functional significance in the MLR response.

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Lymphocytotoxic antibodies produced by H-2 allo-immunisation distinguish between MuLV-positive and -negative substrains of the same H-2 haplotype

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In C57BL mice of certain H-2 types, such as B10.A (H-2^b), ecotropic leukemia virus has been shown to be transmitted by milk-borne infection. By selecting virus-positive and virus-negative B10.A mothers and foster-nursing the B10 (H-2^b) newborn animals, four sub-lines of C57BL/10 mice have been obtained—B10.A V⁺, B10.A V⁻, B10 V⁺ and B10 V⁻ (see Fig. 1 and ref. 1). Milk transmission of naturally prevalent B-tropic virus (V⁺ sub-lines) leads to a persistent infection of all the offspring over more than 8 generations². We have studied the consequences of this kind of virus infection on the expression and immunogenicity of antigens coded for by the H-2 complex. This complex exerts a marked influence on oncogenesis by a variety of oncogenic RNA viruses^{3,4}. The H-2K and H-2D loci restrict the specificity of virus-specific cytotoxic T lymphocytes⁵. In some instances, a direct physical association between H-2 and viral antigens has been demonstrated⁶⁻¹⁰. In our work, groups of B10.A V⁺ and B10.A V⁻ mice were immunised with B10 V⁺ and B10 V⁻ lymphoid cells. Production of anti-H-2^b antibodies was expected and observed in these sera. However, surprisingly, in the combination of B10 V⁺ donor and B10.A V⁻ recipient, after prolonged hyperimmunisation, we obtained sera that reacted positively in the routine microlymphocytotoxicity test with B10.A V⁺ lymph-node cells, but not with B10.A V⁻ cells. B10 V⁻ cells were able to absorb this antibody. Our preliminary conclusion is that the milk-transmitted virus, soon after birth, introduces a serologically detectable antigen which is recognised in association with H-2. The prerequisite for the formation of this kind of antibody is the immunisation of H-2 haplotype-different V⁻ recipients with V⁺ donor cells.

The derivation of sub-lines of C57BL/10 (B10) mice with (V⁺) and without (V⁻) milk-transmitted B-tropic virus is schematically presented in Fig. 1. The individual mice used in the experiments were from the 10th to 12th generation of the respective lines after their original preparation. For immunisations, donor spleen + thymus + lymph-node cells (in 'doses' of 1 donor per 10 recipients) were injected intraperitoneally at weekly intervals with a 1-week interval after every fourth injection. Serum samples were collected every week, 3-4 days after injection or 3-9 days after the last immunisation¹¹. The routine microlymphocytotoxicity test on mouse lymph-node cells (or spleen cells) was carried out in serial geometrical dilutions¹¹. Immunofluorescence tests were carried out using a fluorescein-labelled rabbit anti-mouse IgG serum, prepared in our Institute. Antibodies against AKR type viral-envelope antigens (VEA) were measured by a radioimmunoprecipitation (RIP) test².

The results of four immunisations, carried out in the combination of donor B10, recipient B10.A, are listed in Table 1. All sera exhibited an almost equally strong cytotoxic reaction on cells of the B10 V⁺ and B10 V⁻ substrains. An unexpectedly strong cytotoxic reaction was observed with serum CLS-12 (B10 V⁺ donor, B10.A V⁻ recipient) with the lymph-node (or spleen) cells of B10.A V⁺ mice. More than 20 randomly chosen individual mice of the B10.A V⁺ and B10.A V⁻ substrains, of both sexes, 1-10 months old, were tested. The result was always the same; the individuals V⁺ reacted positively whereas the

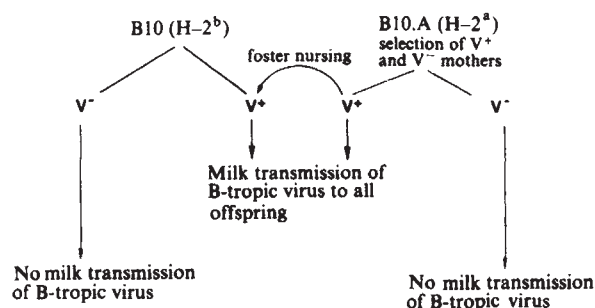


Fig. 1 Derivation of V⁺ and V⁻ sub-lines (see refs 1, 2).

Table 1 Reaction pattern of sera from bleedings during four anti-H-2^b allo-immunisations (CLS-10, -11, -12, -13)

Serum no.	Donor strain and H-2	Recipient strain and H-2	Serum samples tested	Microlymphocytotoxicity titre on cells					
				B10 (bb)*	B10.A(2R) (kb)	B10.A (5R) (bd)	B10.BR (kk)	B10.A V ⁺ (kd)	B10.A V ⁻ (kd)
CLS-10	B10 V ⁻ H-2 ^b	B10.A V ⁻ H-2 ^a	B to Q†	64‡	64	64	0	0	0
CLS-11	B10 V ⁻ H-2 ^b	B10.A V ⁺ H-2 ^a	B to Q	64	64	64	0	0	0
CLS-12	B10 V ⁺ H-2 ^b	B10.A V ⁻ H-2 ^a	B (2)	64	64	64	0	0	0
			C (3)	64	64	64	0	0	0
			D (4)	64	64	64	0	0	0
			F (6)	64	64	64	0	0	0
			G (7)	64	64	64	0	0	0
			H (8)	64	64	64	0	0	0
			I (9)	64	64	64	0	0	0
			J (10)	64	64	64	0	0	0
			K (11)	64	64	64	0	0	0
			L (12)	64	64	64	0	16	0
			M (13)	64	64	64	0	64	0
			N (14)	64	64	64	0	16	0
			O (15)	64	64	64	0	64	0
			Q (17)	64	64	64	0	16	0
CLS-13	B10 V ⁺ H-2 ^b	B10.A V ⁺ H-2 ^a	A to Q	64	64	64	0	0	0

* Strain designation and H-2 alleles at the K and D locus.

† Letters B to Q designate the serial serum sample; the number in parentheses shows the week of immunisation.

‡ Sera were only tested in six dilutions. All reactions with strains B10 V⁺, B10 V⁻, B10.A (2R) and B10.A (5R) were still strongly positive at dilution 1:64.

reaction with V⁻ individuals was negative. Hence, with the routine microlymphocytotoxicity test, simple 'serotyping' of the B10.A V⁺-B10.A V⁻ difference became possible.

The anti-V⁺ activity in the CLS-12 combination developed after about 12 weeks of allo-immunisation. None of the CLS-10, CLS-11 or CLS-13 sera, prepared and tested in parallel, exhibited this kind of activity (Table 1). The data in Table 1 were obtained by testing serum pools obtained from four to six mice from the immunised groups. Because we have shown previously^{11,12} that individual mice of one inbred strain differ widely in their ability to produce anti-H-2 antibodies, the serum samples taken during the last two weeks of immunisation (R + S weeks) were tested as samples of individual mice, not as serum pools. The reactions of the individual CLS-12 sera (10 mice) are shown in Table 2. Of 10 sera, 7 react strongly with B10.A V⁺ cells, but not at all with B10.A V⁻ cells. Two sera exhibited a titre of 1:1,024 with B10.A V⁺ cells.

Absorption experiments (Table 3) have shown that both B10 V⁺ and B10 V⁻ cells absorbed all cytotoxic activity from CLS-12 sera, and that with B10.A V⁺ and control B10.A V⁻ cells, V⁺ cells completely removed activity only against themselves, but left strong activity against the haplotype-specific H-2^b antigens of the donor strain. B10.A V⁻ cells absorbed neither anti-H-2^b nor anti-B10.A V⁺ activity. The observation that B10 V⁻ cells absorbed the anti-B10.A V⁺ activity, whereas B10.A V⁻ cells did not, indicates that MuLV antibodies are not responsible for the reactivity of the CLS-12 sera with B10.A V⁺ cells. First, B10 V⁻ cells are virus negative. Second, B10.A V⁻ cells are virologically identical to B10 V⁻ cells (virus negative) but they do not absorb the activity against B10.A V⁺ lymphocytes. Two other sets of data support the conclusion that anti-MuLV antibodies are not responsible for the anti-B10.A V⁺ activity. Two CLS-12 sera positive with B10.A V⁺ mice were tested in the RIP assay, by far the most sensitive assay for detecting antiviral envelope antibodies, but no such antibodies were found. Moreover, we tested several mouse sera with high titres of antibodies to viral envelope antigens but none of them lysed B10.A V⁺ lymph-node cells. Two of these sera were from 3-month-old B10.A V⁺ mice with high levels of anti-VEA antibodies. Finally, 20 B10.A V⁻ mice were immunised with B10.A V⁺ cells (by an immunisation schedule identical to that applied for the preparation of the CLS-12 sera), but none of these sera became cytotoxic for B10.A V⁺ (B10 V⁺) lymph-node cells. The tests were carried out after 8, 9, 18 and 20 weeks of immunisation; in comparison (Table 1), CLS-12 sera became

positive after 12 weeks of immunisation. This finding shows that only allogeneic V⁺ cells are able to induce cytotoxic anti-B10.A V⁺ antibodies in B10.A V⁻ recipients.

The results described above show a clear-cut difference between the B10.A V⁺ and B10.A V⁻ substrains detected by the lymphocytotoxicity test on normal cells from lymph-node or spleen. These two strains differ only in the presence of a milk-transmitted MuLV, the trait for which they were selected 10-12 generations previously. The serologically defined differences between the two strains might be based on either of two mechanisms. First, a gain mutation might have become fixed in the V⁺ strain and have caused the appearance of a new specificity common to H-2^b and the new H-2 haplotype of B10.A V⁺. Alternatively, a loss mutation in the B10.A V⁻ strain would allow the production of antibodies against a common antigen shared by the H-2^b and H-2^a haplotypes. Both possibilities seem unlikely for two reasons: (1) the anti-B10.A V⁺ antibodies occurred only in the combination defined as CLS-12 (Table 1), and (2) skin grafts survived between the B10.A V⁺ and B10.A V⁻ substrains (data not shown). The second possibility is that the two substrains still differ only in the original trait for which they were selected¹, that is, positive or negative for the milk-transmitted ecotropic MuLV. If this were so, embryos and newborn animals of the B10.A V⁺ substrain should not react with CLS-12 sera, and the reactivity should develop only after drinking mother's milk.

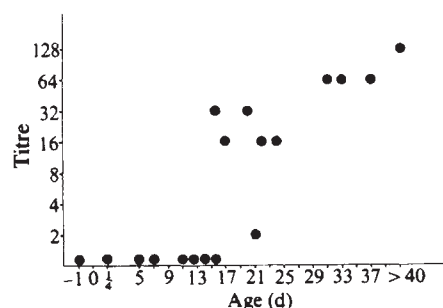


Fig. 2 Postnatal development of positivity with CLS-12 serum (no. 10, R + S bleeding) on spleen cells of B10.A V⁺ mice. The mothers of all litters examined reacted positively (titre 1:64-128). On 1 day before and after birth, the immunofluorescence test was used; on further days, the microcytotoxicity test on spleen cells was used. Titres represent the highest serum dilution with more than 80% killed cells.

The second possibility was examined by testing spleen cells of B10.A V⁺ embryos and newborn mice with CLS-12 serum using immunofluorescence and cytotoxicity tests. Embryos, 1 day before birth, and newborn mice that were maintained 6 ± 3 h after birth without mother's milk, did not react with CLS-12 sera. The cytotoxicity test with CLS-12 sera became positive on spleen cells after day 15 (Fig. 2).

The antibodies in B10.A V⁻ (H-2^a) anti-H-2^b sera which recognise the difference between B10.A V⁺ and B10.A V⁻ sublines of H-2^a mice, have the following characteristics: (1) anti-B10.A V⁺ activity only occurred after immunisation of B10.A V⁻ recipients with B10 V⁺ cells. Such antibodies were not detectable after immunisation with B10 V⁻ cells or after immunisation of B10.A V⁺ recipients. (2) The anti-B10.A V⁺ activity appeared only very late—after more than 10 weeks of allo-immunisation. (3) B10 V⁺ and B10 V⁻ cells each absorbed all activity. (4) B10.A V⁺ cells absorbed only that part of the activity which was directed against the B10.A V⁺ cells, whereas the anti-B10 V⁺ and anti-B10 V⁻ activity remained virtually unchanged. (5) B10.A V⁻ cells absorbed neither the anti-H-2^b nor the anti-B10.A V⁺ activity. (6) The target structure on the B10.A V⁺ cells, responsible for the reaction with CLS-12 sera, developed postnatally, probably as a consequence of virus transmission through milk or placental infection. This target structure develops much later than do H-2 antigens, which are already detectable at birth by cytotoxicity and reach adult sensitivity within 1–3 days¹³.

These characteristics cannot be explained by assuming that the anti-V⁺ and anti-H-2^b activities in CLS-12 sera are due to completely separate populations of antibodies. In that case, B10 V⁻ cells would not be expected to absorb the anti-B10.A V⁺ activity, unless one assumes that B10 V⁻ mice also have the V⁺ antigen but in a form that only absorbs and is not immunogenic. This possibility seems far fetched. Indeed, B10 V⁻ mice express little or no viral structural proteins despite the presence in their genome of latent DNA pro-viruses^{1,2,14,15}. Moreover, B10.A V⁻ cells, that do not differ from B10 V⁻ cells in the lack of expression of infectious virus and/or viral structural proteins^{1,2}, do not absorb the activity against B10.A V⁺. Therefore, it seems likely that all cytotoxic activity of CLS-12 sera is due to anti-H-2^b antibodies, including the anti-B10.A V⁺ activity. On the other hand, the antibody activity against B10.A V⁺ cells constitutes only minor fraction of the anti-H-2^b sera, because after absorption with B10.A V⁺ cells, all activity against B10.A V⁺ is removed, whereas strong anti-H-2^b activity remains.

Having considered all these points, we propose the following explanation for the current observation. Milk-transmitted MuLV induces a lymphocyte antigen (V⁺) which in association with allogeneic H-2^b structures becomes immunogenic in the late phase of B10.A V⁻/anti-B10 V⁺ immunisation. The resulting antiserum cross-reacts with an antigen on normal B10.A V⁺ lymph-node cells (and spleen cells) in the micro-lymphocytotoxicity test. The target antigen on B10.A V⁺ lymphocytes develops within a few days after birth. Whether physical association between V⁺ and H-2 structures is necessary

Table 3 Absorption experiments with CLS-12 (B10 V⁺ donor, B10.A V⁻ recipient) sera

Serum designation	Cells used for absorption	Dose	Cells tested after absorption			
			B10 V ⁺	B10 V ⁻	B10.A V ⁺	B10.A V ⁻
CLS-12-S-8	None		1,024	1,024	1,024	0
	B10 V ⁺	100 × 10 ⁶	0	0	0	0
	B10 V ⁻	100 × 10 ⁶	0	0	0	0
CLS-12-R-7	B10.A V ⁺	100 × 10 ⁶	1,024	0	0	0
	B10.A V ⁻	100 × 10 ⁶	1,024	1,024	1,024	0

Identical results were obtained with two other CLS-12 sera. 50 µl of serum CLS-12 were absorbed with spleen + lymph-node cells.

for immunogenicity remains unclear. The observation that antibody against this antigen was only produced after allo-immunisation in the combination of B10 V⁺ donor, B10.A V⁻ recipient, as well as the finding that B10 V⁻ absorbed all antibody activity, suggests that the immunogen is intimately related to H-2 and possibly represents a virus-altered H-2 antigen.

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Diethylcarbamazine enhances antibody-mediated cellular adherence to *Brugia malayi* microfilariae

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Treatment with the antifilarial drug diethylcarbamazine (DEC) results in a rapid decline in the number of microfilariae circulating in the blood of infected hosts¹. DEC induces morphological changes in the surface layers of microfilariae, but these alterations alone are probably insufficient to cause the death of the parasite, because the drug fails to reduce microfilaraemia in animals lacking filarial antibodies, and also does not shorten the survival of microfilariae *in vitro*^{1–4}. The effect of DEC *in vivo* is thought to result from the trapping of microfilariae in the liver, where they undergo lysis^{1,2,5,6}.

Peripheral blood leukocytes from uninfected donors can be made to adhere to microfilariae of *Brugia malayi* *in vitro*; cellular adherence is promoted by antibodies reacting with the microfilarial sheath, or by a heat labile factor present in normal serum (thought to be complement). The adherence promoting antibody is probably of the IgG class because: (1) only patients' sera that contain high titres of IgG antisheath antibodies

Table 2 Reaction pattern of 10 individual anti-H-2^b (CLS-12) sera

Individual recipients mouse no.	Microlymphocytotoxicity titre on cells*	
	B10A V ⁺	B10.A V ⁻
1	8	0
2	16	0
3	64	0
4	0	0
5	64	0
6	4	0
7	1,024	0
8	1,024	0
9	2	0
10	64	0

The serum samples from individual mice were pools obtained from bleedings on the R(18th) and S(19th) weeks of immunisation.

* All sera had a titre > 1:1,024 on B10 V⁺ and B10 V⁻ cells.

Table 1 Enhancement of antibody-mediated cellular adherence to *B. malayi* microfilariae by diethylcarbamazine (DEC)

DEC concentration ($\mu\text{g ml}^{-1}$)	% Microfilariae with ≥ 5 cells attached				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
0	25 $\pm$ 2	30 $\pm$ 3	15 $\pm$ 2	34 $\pm$ 4	24 $\pm$ 3
100	48 $\pm$ 6	59 $\pm$ 8	35 $\pm$ 5	90 $\pm$ 8	50 $\pm$ 6
10	43 $\pm$ 5	51 $\pm$ 6	32 $\pm$ 4	70 $\pm$ 5	50 $\pm$ 5
5	38 $\pm$ 4	42 $\pm$ 4	32 $\pm$ 5	67 $\pm$ 6	—
1	29 $\pm$ 4	41 $\pm$ 2	30 $\pm$ 3	56 $\pm$ 5	38 $\pm$ 4
0.5	26 $\pm$ 2	32 $\pm$ 3	25 $\pm$ 3	48 $\pm$ 5	—
0.1	24 $\pm$ 3	30 $\pm$ 4	23 $\pm$ 2	46 $\pm$ 4	32 $\pm$ 4

Results shown are means of triplicate determinations $\pm$ s.d. Numbers in italics are significantly different from control values ($P < 0.01$).

detected by indirect immunofluorescence are active in this regard; (2) the adherence reaction is reversed by the addition of low concentrations of staphylococcal protein A which binds to the Fc portion of IgG (unpublished observation); and (3) purified IgG antiseath antibody promotes similar adherence of cells to microfilariae of *Wuchereria bancrofti*⁷. To investigate further the mechanism of action of DEC, we examined the effect of this drug on the adherence reaction. The results indicate that DEC enhances antibody-, but not complement-mediated adherence of buffy coat cells from normal donors to *B. malayi* microfilariae. The effect results from a direct action of the drug on microfilariae and not from a functional activation of leukocytes.

The adherence assay was carried out by sequentially adding the following constituents, each in 0.05 ml volumes to triplicate wells of round-bottom microtitre plates: microfilariae ($4,000 \text{ ml}^{-1}$); diluted antiserum (see below), fresh human serum diluted 1:4 and/or culture medium (Hanks' minimal essential medium, MEM); and buffy coat cells (2×10^5 , prepared by dextran sedimentation of blood from normal volunteers). Serum from three different subjects with elephantiasis due to *B. malayi* was used as the source of IgG microfilarial antibody; these sera were diluted with MEM to promote cellular adherence in the absence of DEC to less than 35% test microfilariae. DEC was dissolved in MEM; the pH of the solution was adjusted to 7.4 with bicarbonate buffer. Adherence was scored after 2 h of incubation at 37 °C.

DEC increased the proportion of microfilariae that formed rosettes with buffy coat leukocytes in the presence of antiseath antibodies in a dose-dependent fashion. The results of five representative experiments in which a wide range of drug concentrations was tested simultaneously are shown in Table 1.

The number of adhering cells per worm was also increased in the presence of DEC, but this phenomenon was difficult to quantitate. In the absence of DEC, 5–20 cells were attached to the surface of microfilariae; in the presence of the drug, most worms were surrounded by several concentric layers of cells, generally exceeding 50 in number. No adverse effects on the

Table 3 DEC increases cellular adherence to microfilariae mediated by sera that are inactive in the absence of the drug

DEC concentration ($\mu\text{g ml}^{-1}$)	% Microfilariae with ≥ 5 cells attached in presence of serum			
	No. 5012	No. 5071	No. 5127	No. 4075
0	3 $\pm$ 1	10 $\pm$ 2	2 $\pm$ 1	7 $\pm$ 1
100	34 $\pm$ 3	27 $\pm$ 3	2 $\pm$ 1	8 $\pm$ 1
10	48 $\pm$ 5	18 $\pm$ 5	1 $\pm$ 1	8 $\pm$ 2

Sera from human patients with Malayan filariasis were used at a dilution of 1:4. Results shown are means $\pm$ s.d. of triplicate determinations. Numbers in italics are significantly different from control values ($P < 0.05$).

motility or viability of microfilariae were observed during the short (2 h) culture period. Microfilariae of *W. bancrofti* are killed under similar experimental conditions, but only after longer periods of incubation⁷.

In preparations stained with Wright–Giemsa, lymphocytes, neutrophils, eosinophils and monocytes were seen to adhere to microfilariae; DEC did not selectively increase the adherence of one or another cell type to the worms.

To determine whether the increased cellular adherence resulted from an effect of the drug on the microfilariae, on the leukocytes or on both, experiments were done in which microfilariae or buffy coat cells were incubated separately with DEC and then washed extensively before they were used in the adherence assay. Preincubation of leukocytes with the drug did not increase cellular adherence to microfilariae. In contrast, preincubation of microfilariae markedly enhanced the proportion of worm-cell rosettes formed in the presence of antibody (Table 2). In seven experiments, complement-mediated adherence of buffy coat cells to microfilariae was not enhanced by DEC added in final concentrations of up to $250 \mu\text{g ml}^{-1}$. Similarly, DEC did not increase the proportion of worms forming rosettes with cells in serum-free medium.

These results clearly indicate that diethylcarbamazine increases the adherence of leukocytes to *B. malayi* microfilariae mediated by antibodies reacting with the surface of the worms. The effect appears to result from a direct action of the drug on microfilariae. However, drug-induced surface alterations *per se* do not promote cellular adherence *in vitro* in the absence of microfilarial antibodies. Likewise, such changes do not promote the *in vivo* hepatic trapping of microfilariae in experimental animals unless antibodies are also present in the treated host⁴. Treatment with DEC of human subjects with patent microfilaraemia rapidly reduces the number of circulating microfilariae. Yet, the serum of such patients contains no antiseath antibodies detectable by indirect immunofluorescence. There are several possible explanations for this apparent discrepancy: it is possible that antiseath antibody titres in microfilaraemia are too low to be detected by rather insensitive immunofluorescent methods; such antibodies may already be bound to the parasites and therefore not readily detectable in serum; or DEC may act *in vivo* by several mechanisms of which only one is measured by the *in vitro* assay used for these studies. In addition, our findings are also consistent with the hypothesis that DEC may act by rendering accessible previously hidden antigenic determinants, antibodies to which might not be detectable in immunofluorescent assays using non drug-treated microfilariae. The observation that some sera from *B. malayi* infected donors fail to promote cellular adherence to microfilariae in the absence of DEC, but enhance adherence in the presence of the drug (Table 3) supports this hypothesis. The mechanism by which DEC unmasks parasite antigens and the exact nature of these antigenic determinants remain to be determined.

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Table 2 Enhanced antibody-mediated cellular adherence following pretreatment of microfilariae with DEC

DEC concentration ($\mu\text{g ml}^{-1}$)	% Microfilariae with ≥ 5 cells attached	
	Worms pretreated	Cells pretreated
0	27 $\pm$ 3	27 $\pm$ 3
100	40 $\pm$ 4	25 $\pm$ 3
10	37 $\pm$ 4	31 $\pm$ 4
1	33 $\pm$ 3	29 $\pm$ 2

Microfilariae ($20,000$) or buffy coat cells (5×10^6) were incubated with the drug concentrations shown for 30 min at 37 °C and washed three times before the addition of leukocytes. Numbers in italics are significantly different from control values ($P < 0.01$). Each datum is the mean $\pm$ s.d. of triplicate determinations.

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Genomic rearrangements correlated with antigenic variation in *Trypanosoma brucei*

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The capacity of African trypanosomes to express sequentially a large repertoire of different surface antigens during an infection¹⁻³ enables the parasite to evade the immune response of its host, and makes attempts to produce a vaccine against the disease difficult. It is evident that point mutations cannot account for antigen diversity^{4,5}. Variable antigens like immunoglobulins are derived from an extensive family of genes of which only one is expressed in a given cell. As somatic recombination is involved in the immunoglobulin gene system⁶, this similarity prompted us to search for somatic rearrangements in trypanosome variable antigen genes. We have constructed a recombinant plasmid containing approximately half the DNA sequence coding for a *Trypanosoma brucei* variable antigen and hybridised the inserted sequences to various restriction enzyme digests of nuclear DNA from different trypanosome clones. Differences in the sizes of restriction fragments hybridising to the inserted variable antigen coding sequence show altered positions of enzyme sites relative to this sequence, indicating different arrangements of DNA sequences around this gene in different trypanosome clones.

Three clones of *T. brucei* expressing different variable antigens were derived from stock LUMP 227 (UHEMBO/64/EATRO/795). Clone A was derived from a relapse infection of clone C. Clone B was derived from a relapse infection of clone A. Clone X was derived from a different trypanosome stock, S427. All trypanosome populations, grown in lethally irradiated rats for these studies, were monitored for variable antigen type homogeneity by immunofluorescent techniques⁷.

Messenger RNA was prepared from *T. brucei* clone A, expressing variable antigen ILTat 1.2, as described previously⁸. A preparation enriched for variable antigen mRNA was obtained by two cycles of density gradient centrifugation. Double-stranded cDNA was made on this template by a slight modification of the procedure of Wickens *et al.*⁹, and inserted into the plasmid vector pBR322 by the G-C boundary method¹⁰. Transformed bacteria containing recombinant plasmids were screened by *in situ* filter hybridisation¹¹. Isolated plasmids were further screened for variable antigen coding sequences by hybrid-arrested translation¹². Thus it was shown that plasmid pcB602 contains variable antigen coding sequences (Figs 1 and 2).

A filter hybridisation of the inserted sequences of pcB602 to *Hind*III fragments of nuclear DNA from four different *T. brucei* clones is shown in Fig. 3. In all clones there is a strongly hybridising fragment. It is the same size in clones B and C (4.5 kilobases) but slightly larger (5.0 kilobases) in clone A, this clone expressing the sequences in the probe. In clones B and C

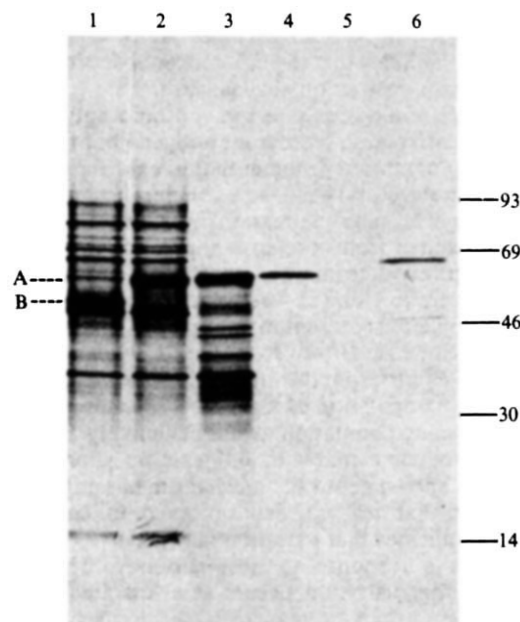


Fig. 1 Identification of variable antigen band in SDS polyacrylamide gel electrophoresis of *in vitro* translation products. Translation products of total mRNA from *T. brucei* clones: lane 1, clone B; lane 2, clone A; lane 3, translation products of partially enriched variable antigen mRNA from clone A. Translation products of total mRNA from clone A immunoprecipitated with: lane 4, antiserum against clone A antigen; lane 5, with normal rabbit serum. Lane 6, immunoprecipitate of variable antigen from *in vivo* labelled trypanosome with anti-clone A variable antigen antiserum. Antiserum was raised against purified variable antigen²⁰ by conventional procedures. Polyadenylated RNA was further purified by two cycles of sucrose density gradient centrifugation. Translation products were made in the rabbit reticulocyte cell-free system²¹. Solubilised extracts of ³⁵S-methionine-labelled proteins from the cell free translation reactions and from trypanosomes labelled *in vivo* (S. Z. Shapiro, unpublished) were made by the procedure of Shapiro and August²² and immunoprecipitated by the procedure of Kessler²³. Electrophoresis was performed with a 7.5-17.5% polyacrylamide gradient slab gel containing 0.1% SDS using the discontinuous buffer system of Maizel²⁴. Position of *in vitro* synthesised variable antigen bands are indicated at A (clone A) and at B (clone B). The former is identified by immunoprecipitation. It has a lower apparent molecular weight (60,000) than the glycosylated *in vivo* labelled protein (63,000) as expected⁸.

there is a less strongly hybridising fragment of very different size in each clone, 7 kilobases in clone B and 8.5 kilobases in clone C. In clone A, there is weaker hybridisation to three fragments, 11, 8 and 6 kilobases long.

Plasmid pcB602 has been sequenced (K. Chen, J. E. Donelson and R. O. W., unpublished work). It contains 902 base pairs of coding sequence. There is a single *Hind*III site which is 293 base pairs from one end. If the same site were present in genomic DNA we would expect to find at least two genomic *Hind*III fragments hybridising to the two segments of the probe sequence. This would be consistent with what is seen with clones B and C. With clone A there are three weakly hybridising fragments. This could result from the presence of *Hind*III sites in very long intervening sequences, or asymmetric length heterogeneity of multiple gene copies. There is no evidence concerning gene reiteration.

Using nuclear DNA from clone X, some hybridisation to a fragment of very different size from the major fragments observed in the other clones (13 kilobases) was detected. This result shows that this clone contains a genomic sequence of sufficient homology to the clone A antigen coding sequence to hybridise in these conditions. In similar experiments using genomic DNAs digested with the restriction enzymes *Hinc*II,

HinfI and *XhoI* differences in hybridisation patterns were observed between trypanosome clones A, B, C and X in all cases. Thus it is unlikely that the differences observed are due to point mutations in restriction enzyme sites.

The sequence coding for one trypanosome antigen is conserved in clones expressing different antigens but these clones have differently organised genomes in the region containing the clone A antigen structural gene. Are structural changes responsible for change in gene expression? We have found that a trypanosome cloned from a relapse population has an altered genomic structure. We cannot exclude the possibility that the genomic structure in clone B was selected fortuitously by the cloning of a single unrepresentative organism from the relapse population of clone A. However, neither weaker nor stronger bands in clone B correspond to those in clone A. The cloning from the relapse population of a genomic structure not detectable in the infecting population would be unlikely unless structural changes are correlated with antigenic variation.

Very little is known about the mechanism of antigenic variation. The fact that it has been observed in cultured trypanosomes¹³ indicates that variation may occur spontaneously rather than as a response to host antibody. The simplest assumption is that variation occurs at a low frequency in a

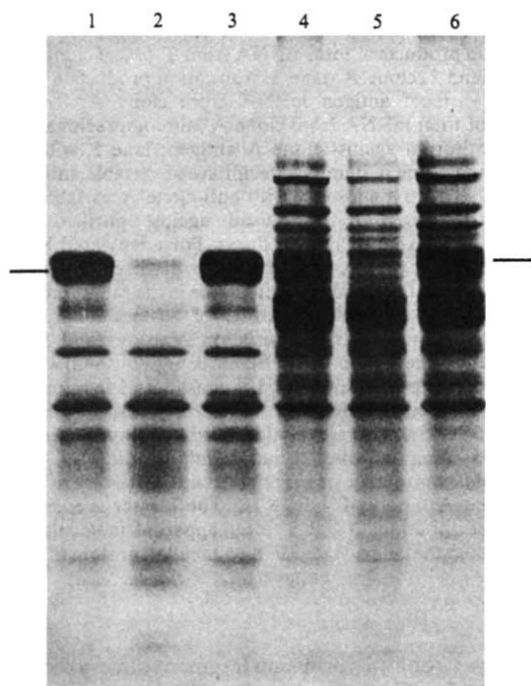


Fig. 2 Hybrid-arrested translation confirming that pcB602 contains variable antigen coding sequences. Translation products of partially enriched variable antigen mRNA; lane 1, unhybridised; lane 2, hybridised with pcB602; lane 3, hybridised with pcB602 then denatured by boiling. Translation products of total mRNA: lane 4, unhybridised; lane 5, hybridised with pcB602; lane 6, hybridised with pcB602 then denatured by boiling. Hybridisations, carried out according to Paterson¹², contained in a final volume of 50 μ l, 10 μ g of *HaeIII*-digested pcB602 and 1 μ g of enriched mRNA fraction or 2 μ g *HaeIII*-digested pcB602 and 1 μ g total mRNA. After 4 h at 48 °C, 100 μ l of cold water was added and the mixture was divided. One aliquot was boiled for 90 s, before both were ethanol precipitated and translated in the rabbit reticulocyte system. Electrophoresis of translation products was through a 10% polyacrylamide SDS slab gel. The variable antigen band as identified by immunoprecipitation is marked by the horizontal bars. Specific and reversible inhibition of synthesis of this polypeptide by hybridisation with pcB602 shows that the plasmid contains variable antigen coding sequences. Control experiments with pBR322 and another recombinant plasmid containing G-C boundaries gave no inhibition of synthesis of this band.

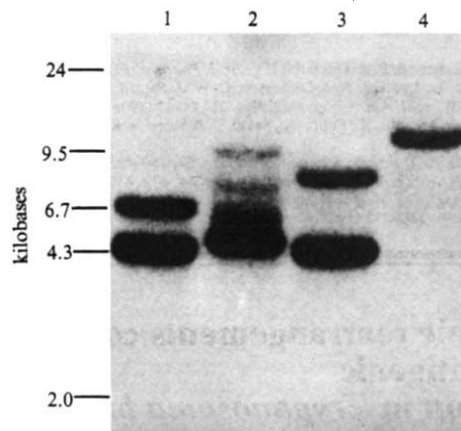


Fig. 3 Hybridisation of inserted sequences of pcB602 to *HindIII* fragments of nuclear DNA: lane 1, clone C; lane 2, clone A; lane 3, clone B; lane 4, clone X. Plasmid pcB602 was digested with *PstI*. The three fragments of the inserted sequence were separated by electrophoresis, recovered from an agarose gel²⁵ and labelled with ³²P by nick translation²⁶ to high specific activity ($\geq 250 \times 10^6$ ³²P c.p.m. μ g⁻¹). Nuclear DNAs were prepared from cloned trypanosomes using a modification of published procedures²⁷. DNA was completely digested with the restriction enzyme *HindIII* and electrophoresed in 0.6% agarose gels. Fragments were transferred to nitrocellulose filters by the blotting procedure of Southern²⁸, and hybridised to the nick translated probe (in 120 mM Tris HCl, pH 7.4, 4 mM EDTA, 0.6 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 0.2% each of Ficoll 400, polyvinyl pyrrolidone and bovine serum albumin, and 100 μ g ml⁻¹ sonicated denatured calf thymus DNA) at 68 °C for 60 h. After extensive washing the filters were autoradiographed using Kodak XR5 film with intensifying screens.

trypanosome population, and that new variants become apparent in successive waves of parasitaemia as the preceding ones are eliminated by the host's immune system³. If this is an accurate interpretation, then we are observing an alteration of gene expression which is stably inherited by the vast majority of progeny cells. The process resembles differentiation in higher eukaryotes more than transient forms of control of gene expression, such as hormonal regulation. Thus antigenic variation may provide an important model for some general processes in eukaryotic differentiation.

Our data do not allow us to distinguish between the many possible forms of DNA rearrangement that could account for the changes observed. Immunoglobulin genes are known to be constructed by movement of variable region genes into proximity with the constant region gene⁶. While trypanosome variable antigens do exhibit general immunological cross-reactivity¹⁴, it has not yet been determined whether this involves regions of polypeptide homology or the attached carbohydrate structures (A. F. Barbet, personal communication). Therefore, we cannot implicate an analogous mechanism in construction of variable antigen genes. In prokaryotes translocatable genomic elements are known to exert control over gene expression¹⁵⁻¹⁷. Somewhat analogous translocating element hypotheses have been used for eukaryotes to explain some phenomena in the genetics of maize¹⁸ and *Drosophila*¹⁹. Similar mechanisms might possibly occur in trypanosomes.

We have observed differences in genomic structure in the region containing sequences coding for the clone A variable antigen in different clones of *T. brucei* expressing this and two other antigens. We propose that the rearrangements observed may be involved in controlling the expression of this gene. A sequence with some homology to that coding for clone A variable antigen is also present in a clone from *T. brucei* stock S427.

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Table 1 Inhibition of DNA synthesis in Con A-stimulated mouse spleen lymphocytes by interferon and by (2'–5') oligo-isoadenylate

Addition to Con A-stimulated cultures	Time of addition	DNA synthesis at 72 h ³ H-thymidine incorporated, c.p.m.	Inhi- bition (%)
Experiment 1			
None	—	111,020	0
Interferon, 200 U ml ⁻¹	0 h	13,175	88
Interferon, 200 U ml ⁻¹	24 h	66,050	41
Interferon, 200 U ml ⁻¹	48 h	102,060	8
Experiment 2			
None	—	66,150	0
(2'–5')ApApApA, 5 µM	0 h	17,665	74
(2'–5')ApApApA, 5 µM	24 h	18,800	72
(2'–5')ApApApA, 5 µM	48 h	56,765	15

Mouse spleen lymphocytes (8×10^6 cells per ml) were treated with $2.5 \mu\text{g ml}^{-1}$ Con A. Interferon (200 U ml^{-1}) or (2'–5')ApApApA ($5 \mu\text{M}$) were added 10 min later (zero time) or at various times after Con A. DNA synthesis was measured at 72 h by a 3-h pulse of $6 \mu\text{Ci ml}^{-1}$ ³H-thymidine (5 Ci mmol^{-1}). Cells were then collected on glass fibre filters (Whatman GFC), washed twice with 5 ml cold PBS, twice with 5 ml 5% TCA then with 95% ethanol, dried and counted. ³H-thymidine incorporated into the unstimulated lymphocytes ($28,750 \text{ c.p.m.}$) was subtracted from that incorporated into the mitogen-treated cells.

The mitogenic stimulation of mouse spleen lymphocytes by concanavalin A (Con A) was studied. Spleens of BALB/c mice (8–10 weeks old) were teased through a metal mesh and cells were suspended in Dulbecco's phosphate-buffered saline (PBS). Erythrocytes were lysed in 0.01 M KHCO_3 , $0.155 \text{ M NH}_4\text{Cl}$ and 0.1 mM EDTA , and cells were washed twice with PBS. Lymphocytes were cultured at $4\text{--}8 \times 10^6$ cells per ml in RPMI 1640 medium (Gibco), or when indicated in DMEM, supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$). Cultures of 1 ml were incubated at 37°C , in 5% CO_2 –95% air (in $17 \times 100 \text{ mm}$ plastic tubes, Falcon No. 2051). Con A (Miles Yeda) was used at $2.5 \mu\text{g ml}^{-1}$ which gave optimal stimulation of ³H-thymidine incorporation. Mouse L cell interferon was prepared and purified partially to $10^7 \text{ U per mg protein}$, as previously described¹⁶.

In Con A-stimulated lymphocytes, DNA synthesis started at 48 h and was maximal by 72 h. Interferon added immediately after Con A, strongly reduced DNA synthesis measured at 72 h. The dose of interferon that caused 50% inhibition of thymidine incorporation ranged from 130 to 250 units ml^{-1} in several experiments. The inhibitory effect of interferon on subsequent DNA synthesis gradually decreased if interferon was added after the cells had been exposed to Con A (Table 1). In this experiment, when interferon (200 U ml^{-1}) was added by 24 h, the inhibition was only half of that measured at zero time, and by 48 h the inhibition was very small.

The activity of the (2'–5') oligo(A) synthetase E was studied in stimulated lymphocytes, in conditions where interferon strongly inhibits subsequent DNA synthesis. Cytoplasmic extracts were prepared with NP40 and (2'–5') oligo(A) synthetase measured after binding to poly(rI)·(rC) agarose. Table 2 shows that 24 h after adding interferon to unstimulated resting lymphocytes, as well as to Con A-stimulated cells, the activity of the (2'–5') oligo(A) synthetase is increased. The basal activity without interferon was higher in these cells than that in L cells¹⁴, but there was no difference in the basal level of unstimulated and mitogen-stimulated lymphocytes. The double-stranded RNA-dependent eIF-2 kinase $\text{P}_k\text{-i}^{12}$ was also clearly induced in the interferon-treated lymphocytes (not shown).

As interferon induces an increase in (2'–5') oligo(A) synthetase E in the lymphocytes, we determined whether exogenously added (2'–5') oligo(A) could mimic the inhibitory effect of interferon on DNA synthesis. Because highly charged

Regulation of lymphocyte mitogenesis by (2'–5') oligo-isoadenylate

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Interferon has been reported to affect cell proliferation in various tumour and normal cell cultures¹. In human cells, the anti-growth effect of interferon is under the control of chromosome 21, like the antiviral effect^{2,3}, and the two activities appear to reside in the same glycoprotein⁴. In synchronised fibroblast cultures interferon inhibited DNA synthesis by blocking initiations of DNA replication^{5,6}. Another system in which the inhibitory effect of interferon on DNA synthesis was particularly clear is lymphocyte mitogenesis. In lymphocytes stimulated by lectin mitogens or allogeneic cells, interferon inhibits DNA synthesis, and the RNA and protein synthesis observed before the S phase of the cell cycle^{7–9}. In interferon-treated cells, several enzymes are induced, including the (2'–5') oligo-isoadenylate [oligo(A)] synthetase E, which polymerises ATP into a series of oligonucleotides of general structure $\text{ppp}(\text{A}_2\text{p})_n\text{A}$ (refs 10–12). The (2'–5') oligonucleotides inhibit protein synthesis when added to cell-free systems^{10–12} or hypotonically permeabilised cells¹³, and the induction of (2'–5') oligo(A) synthetase was correlated with the antiviral action of interferon^{14,15}. In the present work, the involvement of this enzyme in the antigrowth effect of interferon was studied. We show that addition of the (2'–5') oligo(A) nucleotides to the culture medium of intact lymphocytes mimics the antimitogenic effect of interferon. Experiments with lymphocyte extracts show that, while interferon increases the (2'–5') oligo(A) synthetase E, the mitogenic stimulus of concanavalin A leads to a decrease in (2'–5') oligo(A) accumulation.

Table 2 (2'-5') Oligo-isoadenylate synthetase E activity in mouse spleen lymphocytes

	Poly (rI) : (rC) bound fraction from	Oligo-isoadenylate synthesis [³² P]-(2'-5')ApA (c.p.m.)
Expt 1	Untreated cells	3,315
	+ interferon 200 U ml ⁻¹	7,785
	+ interferon 100 U ml ⁻¹	12,117
Expt 2	Untreated cells	1,225
	+ Con A 2.5 g	1,305
	+ Con A + interferon 1000 U ml ⁻¹	3,910
	+ interferon, 1000 U ml ⁻¹	2,590

Lymphocyte cultures ($4-8 \times 10^6$ cells per ml) in DMEM were treated for 24 h as indicated. 4×10^7 cells (expt 1) or 2.4×10^7 (expt 2) were washed twice with 35 mM HEPES buffer pH 7.5, 140 mM KCl, 3 mM MgCl₂ and lysed in 0.1 ml of 20 mM HEPES buffer pH 7.5, 120 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol (buffer B) containing 0.5% Nonidet P40 (NP40). Lysates were centrifuged for 6 min in an Eppendorf centrifuge (8,000g) and 20–30 µl supernatant (expt 1, 16 µg protein; expt 2, 8 µg) mixed with 25 µl packed poly(rI)·(rC)-Agarose beads (PL Biochemicals) for 5 min at room temperature. The non-adsorbed material was removed by centrifugation, the pellet washed with buffer B and 10 µl of 2.5 mM [³²P]ATP (3 Ci mmol⁻¹) and 2.5 mM dithiothreitol were added. The suspension was incubated 20 h at 30 °C and 6 µl of the supernatant was treated 30 min at 37 °C with 0.2 units of BAP in 30 mM Tris base. 3 µl were applied to thin layer sheets of polyethylenimine (Schleicher and Schull) and chromatographed in 0.5 M acetic acid. Enzyme E activity was determined as before¹² by counting the ³²P-(2'-5')ApA formed.

nucleotides would not penetrate intact cells, we tried the (2'-5') oligo(A) without 5' phosphates, that is the bacterial alkaline phosphatase (BAP) resistant nucleotide cores. A series of oligomers (chemically synthesised by Rapoport and Lapidot¹⁷) were added to the medium of Con A-stimulated lymphocytes. Table 3 shows that (2'-5')ApApA or longer oligomers (tetramer and pentamer) efficiently inhibit the Con A-stimulated DNA synthesis. Concentrations of $2-5 \times 10^{-6}$ M were effective but below 1 µM the trimer had little effect on DNA synthesis in these intact lymphocytes. The effect is specific since the (3'-5')ApApA is devoid of inhibitory activity (Table 3). Williams and Kerr¹³ showed that the BAP resistant cores of (2'-5')pppApApA were able to inhibit ³⁵S-methionine incorporation in permeabilised cells, whereas in cell-free systems of protein synthesis these BAP-treated nucleotides were inactive¹⁰. Possibly, the intact cell can convert the cores into the active form by phosphorylation, a function which is lost in cell-free systems.

Adding (2'-5')ApApApA to lymphocytes 24 h after Con A stimulation was as effective as at time zero, whereas at 48 h or later the oligonucleotide did not inhibit subsequent DNA synthesis (Table 1). This suggests that (2'-5') oligo(A) inhibits some events preceding entry into S phase.

The above experiments suggest that the increase in (2'-5') oligo(A) synthetase E produced by interferon treatment, could mediate the inhibition of DNA synthesis in stimulated lymphocytes. When (2'-5') oligo(A) synthetase E was measured after isolating the enzyme from the cell extract with poly(rI)·(rC)-Agarose, Con A treatment neither reduced the basal level of the enzyme in lymphocytes nor its induction by interferon (Table 2). However, Table 4 shows that extracts of Con A-stimulated lymphocytes contain an activity that inhibits the synthesis of (2'-5') oligo(A) if added to a known amount of enzyme E. Thus, the amount of (2'-5') oligo(A) formed by the enzyme was much smaller in the presence of extracts from Con A-stimulated lymphocytes than with those from lymphocytes maintained without mitogen. Fresh splenic lymphocytes (time zero in Table 4) also contain the inhibitor. The level of inhibitor of (2'-5') oligo(A) synthesis is, therefore, high in lymphocytes in which DNA synthesis is active: fresh splenic lymphocytes synthesise DNA and this decreases rapidly unless a mitogen is added to the

culture¹⁸. Similarly, the level of inhibitor of (2'-5') oligo(A) formation decreases when the lymphocytes are maintained in culture without mitogen, but increases if Con A is added. The differential inhibition of (2'-5') oligo(A) synthesis was maximum at 72 h after Con A stimulation, but was already marked at 48 h and even 24 h after initiating the cultures (Table 4). The nature of the inhibitor is unclear: it is heat labile and non-dialysable (not shown). Preliminary results show that extracts of Con A-stimulated lymphocytes contain an activity which degrades the oligonucleotide formed, as does the 2'-phosphodiesterase described previously^{19,20}.

The Con A mitogenic stimulus is, therefore, accompanied by a high level of an inhibitor of (2'-5') oligo(A) synthesis, which could lower the basal rate of oligonucleotide formation and allow entry into S phase. This is supported by the fact that exogenous (2'-5') oligo(A) addition inhibits entry into S phase. The effect of interferon on Con A-stimulated lymphocytes may be explained by the induction of the (2'-5') oligo(A) synthetase E to a level which exceeds the activity of the inhibitor (the latter remained unchanged after interferon; not shown). Since cellular

Table 3 Effect of different oligonucleotides on DNA synthesis in Con A-stimulated mouse spleen lymphocytes

Oligo(A) added	Final concentration (µM)	Inhibition of incorporation of ³ H-thymidine (%)
None	—	0
(2'-5') trimer	2	81
A2'p5'A2p5'A	5	88
	10	87
(3'-5') trimer	5	0
A3'p5'A3'p5'A	10	0
(2'-5') tetramer	1	0
A2'p5'A2'p5'A2'p5'A	5	50
	10	73
(2'-5') pentamer	5	64
A2'p5'A2'p5'A2'p5'A2'p5'A	10	94

Mouse spleen lymphocytes (8×10^6 cells per ml) were treated with $2.5 \mu\text{g ml}^{-1}$ Con A and 10 min later the different oligonucleotides (chemically synthesised as in ref. 17) were added. DNA synthesis was measured at 72 h as described in Table 1. The total ³H-thymidine incorporated into TCA insoluble material of 1 ml suspension in the absence of any oligonucleotide was 125,000 c.p.m. for the stimulated lymphocytes. Per cent inhibition was calculated after the values of unstimulated cells (32,800 c.p.m.) was subtracted.

Table 4 Inhibition of the (2'-5') oligo-isoadenylate synthetase in Con A-stimulated mouse spleen lymphocytes

Lymphocyte extracts prepared at:	Inhibition of (2'-5') oligo(A) synthesis	
	No mitogen (%)	Con A (%)
0 h	60*	—
24 h	45	60
48 h	15	60
72 h	15	80

Extracts (0.1 ml) from 5×10^7 Con A-stimulated lymphocytes or 10^8 untreated cells were prepared at 0, 24, 48 and 72 h as in Table 2. Cell viability in the unstimulated culture was high (>90%) throughout the experiment as determined by staining with Trypan blue. The inhibitor of (2'-5') oligo(A) accumulation was measured as follows: Insoluble (2'-5') oligo(A) synthetase was prepared by mixing 165 µg protein from the 0.5 M KCl ribosome extract from interferon-treated L cells¹² with 0.3 ml packed poly(rI)·(rC)-Agarose as in Table 2. To 20 µl packed beads, 6 µl of 1.5 mM [α -³²P]ATP (3 Ci mmol⁻¹) and 10 µl of various concentrations of lymphocyte extracts (5–15 µg) were added. After 2 h at 30 °C the synthesis of (2'-5') oligo(A) was measured as in Table 2. The inhibition was calculated for 8 µg protein extract. Without extract 2,570 c.p.m. of ³²P-(2'-5')ApA was formed.

* Fresh splenic lymphocytes before culture.

RNAs activate enzyme E¹⁷, interferon could lead to accumulation of the oligonucleotides and, as found for exogenously added (2'-5') oligo(A), inhibit entry into S phase. It will be interesting to establish whether the inhibitor of (2'-5') oligo(A) synthesis, described here, exists in other cells and is increased in fast growing cells. It will also be important to determine whether the antimetogenic effect of exogenous (2'-5')ApApA results from phosphorylation of the oligonucleotide in the cell and activation of RNase F^{20,21} or from some other function of these oligonucleotides. Finally, the (2'-5') oligo(A) may become useful to regulate the immune response. [Recently, E. Martin (personal communication) reported that the (2'-5')ApApA also inhibits DNA synthesis in cultures of lymphoblastoid Daudi cells.]

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Ionomycin stimulates mast cell histamine secretion by forming a lipid-soluble calcium complex

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The activation of cellular function following the direct introduction of Ca²⁺ into the cytosol by the use of a Ca²⁺-transporting ionophore has served to confirm the widely held idea that Ca²⁺ has the status of a second messenger in many cell types^{1,2}. However, this evidence has been obtained largely from the use of a single ionophore, the antibiotic A23187; experiments with X537A, which is another ionophorous antibiotic capable of transporting Ca²⁺ (ref. 3), have failed to show the expected characteristics. For example, histamine release from rat mast cells mediated by X537A is neither dependent on extracellular Ca²⁺ nor prevented by metabolic inhibitors⁴. Ionomycin is a recently described polyether antibiotic produced by *Streptomyces conglobatus* ATCC 31005, and is active against Gram-positive bacteria⁵. The antibiotic action is presumably due to its ionophorous properties, as it extracts Ca²⁺ from an aqueous phase into an organic phase with a stoichiometry of 1:1 (ref. 6). The ionophore is also capable of transporting ⁴⁵Ca²⁺ across biological membranes (our unpublished results). Here we report the application of ionomycin to rat mast cells. We show that ionomycin stimulates mast cell secretion solely through its ability to form a lipid-soluble calcium complex, and thus to convey Ca²⁺ across the hydrocarbon region of the cell membrane.

Ionomycin-mediated histamine secretion is completely dependent on the presence of extracellular Ca²⁺, although, as shown in Fig. 1, the actual concentration of Ca²⁺ required varies inversely with the concentration of the ionophore used. The secretion seems to occur by the normal exocytotic mechanism, as the cells show no loss of the cytoplasmic marker enzyme, lactate dehydrogenase (<5% at 5 µM ionomycin), and secretion is prevented when cell metabolism is inhibited by preincubation for 10 min with antimycin (5 µM) in the absence of glucose.

The partial secretions induced at suboptimal concentrations of Ca²⁺ in the curves of Fig. 1 represent completed secretory events; they are not the consequence of reduced rates of exocytosis. Secretion at different suboptimal ionophore concentrations occurs at similar rates with t_{1/2} ~ 0.6 min and completion by 6 min (Fig. 2). At higher ionomycin concentrations (2.5 µM) secretion stops abruptly when the histamine which is available for secretion (normally about 80% of total) is exhausted.

There was little further secretion on treatment with additional ionomycin after secretion due to a first suboptimal dose was complete; this suggests that the characteristic time course for cessation of secretion reflects termination by some cellular mechanism. As shown in Fig. 2, application of 1.5 µM ionomycin in two stages (each of 0.75 µM) caused rather less secretion than 1.0 µM ionomycin at a single application. Such characteristics might result from a decrease in the sensitivity to internalised Ca²⁺ following its initial elevation due to the ionophore (for example, by the recruitment of additional Ca²⁺-pumping or Ca²⁺-buffering capacity) or by a termination mechanism operating at a subsequent stage in the Ca²⁺-initiated sequence of events which lead to secretion.

Of the Ca²⁺ present in the extracellular medium, only that fraction which is present as a calcium-ionomycin complex will be able to partition into the cell membrane and thence into the cytoplasm. To estimate the size of this fraction, we used an organic extraction technique^{7,8} to measure the equilibrium



H₂I and I²⁻ represent the protonated and fully dissociated forms of ionomycin, respectively. The dependence on the aqueous Ca²⁺ concentration of the amount of ⁴⁵Ca²⁺ extracted into an organic phase is shown in Fig. 3.

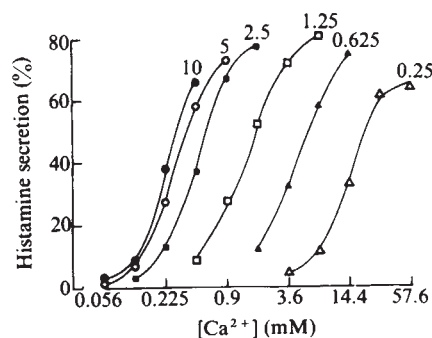


Fig. 1 The concentration dependence on Ca²⁺ of histamine secretion due to different concentrations of ionomycin. Rat peritoneal mast cells were isolated and purified as previously described¹⁵ and suspended at ~10⁵ cells per ml in a buffered salt solution, pH 7.5, comprising 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 10 µM EGTA, 20 mM HEPES, 5.6 mM glucose and 1.0 mg ml⁻¹ bovine serum albumin. Solutions were equilibrated to 37 °C before addition of 0.2 ml cells to tubes containing ionomycin and CaCl₂ to give the indicated concentrations in a final volume of 0.5 ml. Secretion was terminated after 10 min by the addition of 1.0 ml ice-cold phosphate-buffered saline and the cells were centrifuged at 4 °C. The histamine content in the supernatants was determined fluorimetrically¹⁵ and is expressed as a percentage of total cell histamine. Data points represent the means of duplicate determinations which were within 10% of each other. Concentrations of ionomycin (µM) are indicated above each concentration-effect curve.

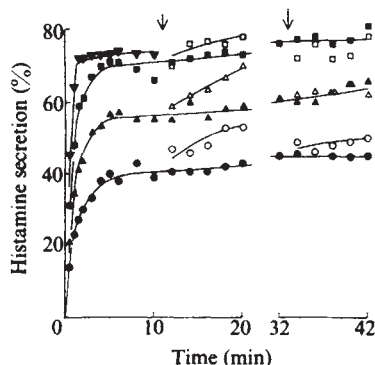


Fig. 2 Time courses of ionomycin-induced secretion. Secretion was initiated by adding 5 ml cells ($\sim 1.5 \times 10^5$ cells per ml) to buffer containing CaCl_2 (final concentration 1.8 mM) and ionomycin, giving a total volume of 20 ml. At the times indicated by vertical arrows, 3 ml was transferred to another tube containing additional ionomycin (in less than 10 μl) sufficient to double the concentration. Samples (0.5 ml) from these incubations were quenched at different times by addition to 1.0 ml ice-cold phosphate-buffered saline and the extent of histamine secretion was determined as in Fig. 1. Curves show time courses of secretion due to ionomycin at: $\bullet$, 0.75 μM ; $\blacktriangle$, 1.0 μM ; $\blacksquare$, 1.25 μM ; $\blacktriangledown$, 2.5 μM . Additional secretion due to doubling of ionomycin after the initial secretion was complete is shown: $\circ$, 0.75–1.5 μM ; $\triangle$, 1.0–2.0 μM ; $\square$, 1.25–2.5 μM .

The solubility of inorganic ions in low dielectric organic phases is extremely small (ref. 1, p. 45), so the initial stage in the complexation by ionomycin is likely to occur in the aqueous phase; this can be concluded from kinetic studies⁹ and from the use of fluorescent probe techniques¹⁰ in the analogous reaction of alkali-metal ionophores. As evidence for the existence of the complex in the aqueous phase, we find a Ca^{2+} -dependent change in the UV spectrum of aqueous ionomycin, with an absorption maximum at 305 nm for the difference spectrum. Equation (1) can therefore be considered as the sum of four component equilibria

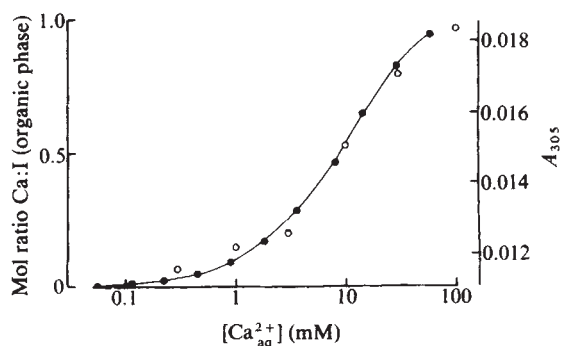


Fig. 3 Extraction of Ca^{2+} into organic phase by ionomycin. The transfer of radioactivity from an aqueous phase (the buffered salt solution described in Fig. 1 legend) containing different concentrations of $^{45}\text{CaCl}_2$ ($\sim 3 \times 10^6$ c.p.m. μmol^{-1}) into an equal volume of organic phase (toluene/butanol, 7:3) containing 20 μM ionomycin was measured as previously described⁸. The extraction of Ca^{2+} is expressed as a mol fraction of the ionomycin present (solid symbols). The figure also shows the dependence on calcium concentration of the spectral change of aqueous ionomycin. The absorbance at 305 nm was measured for ionomycin (1 μM) in 1 ml buffered salt solution (as described in Fig. 1 legend, but omitting MgCl_2 , which also caused a spectral change, and albumin, which absorbs at this wavelength) containing different concentrations of CaCl_2 . Higher concentrations of ionomycin could not be used because at the highest calcium concentrations visible precipitation occurred in the cuvette.

Equations (2) and (5) represent the partition coefficients of ionomycin and its calcium complex; the aqueous equilibrium is described by equations (3) and (4), with equation (3) representing the acid dissociation of ionomycin, which remains constant throughout our experiments (because we work at constant pH). The two experiments described by Fig. 3 show that the extent to which ionomycin extracts Ca^{2+} into an organic phase (closed symbols, equation (1)) is indicative of the aqueous equilibrium between the free acid and Ca^{2+} (open symbols, equation (4)), as both reactions show a similar dependence on aqueous phase Ca^{2+} .

In the buffered salt solution used in our experiments, ionomycin has a rather low affinity for Ca^{2+} (Fig. 3). Ionomycin has $pK_a \sim 8.3$ (ref. 6) so that in our buffered salt solution (pH = 7.5), protonation of ionomycin (equation (3)) occurs in preference to the formation of the calcium complex (equation (4)). The affinity for Ca^{2+} is very much higher at pH 10 (ref. 6). Using the data of Fig. 3 we have estimated the aqueous phase calcium-ionomycin concentration for the data points of Fig. 1 (and for another similar experiment) and the histamine secretion is plotted in Fig. 4 as a function of calcium-ionomycin.

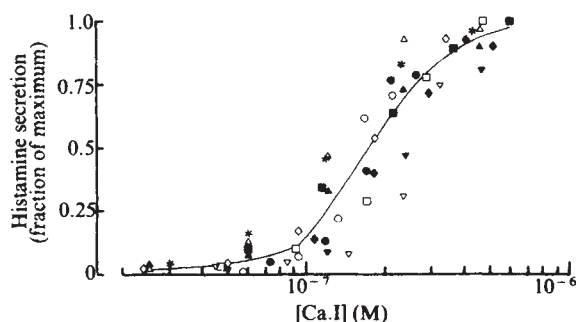


Fig. 4 The dependence of histamine secretion on the calculated concentration of calcium-ionomycin complex. The fraction of ionomycin which was present as the calcium-complex at a given CaCl_2 concentration (Fig. 3) was used to determine the calcium-ionomycin concentration for the data points of Fig. 1 (solid symbols) and another similar experiment (open symbols). For each experiment histamine secretion was normalised as a fraction of the maximum secretion observed. Total ionomycin concentrations were: $\blacktriangledown$, 10 μM ; $\blacktriangle$, $\triangle$, 5 μM ; $*$, 2.5 μM ; $\diamond$, 2 μM ; $\blacksquare$, 1.25 μM ; $\square$, 1.0 μM ; $\blacklozenge$, 0.625 μM ; ∇ , 0.5 μM ; $\bullet$, 0.25 μM ; $\circ$, 0.2 μM .

The assumption that ionomycin-induced histamine secretion can be attributed to the aqueous concentration of calcium-ionomycin is supported by the data in Fig. 4. Concentration-effect curves falling over a 70-fold range of Ca^{2+} concentrations (Fig. 1) are encompassed within a twofold scatter about a curve which indicates cell stimulation over a calcium-ionomycin range of 0.1–0.3 μM . Although calcium-ionomycin has access to the cell interior, these concentrations do not represent the range over which cytoplasmic Ca^{2+} will be elevated. At the low concentrations of ionised Ca^{2+} believed to exist in the cytoplasm¹¹, calcium-ionomycin will rapidly and completely dissociate as soon as it enters the cell, and it is the rate of this partition process which will be a function of the external aqueous calcium-ionomycin concentration.

Our results indicate that it is the rate of entry of calcium into the cell which controls the extent (and not kinetics) of secretion. For normal (receptor-mediated) secretion, this implies that the extent of secretion will depend on the number of Ca^{2+} channels, and thus, presumably, on the number of stimulated receptors. The consequent intracellular signal could be an elevated concentration of cytoplasmic Ca^{2+} , occurring as a result of the relative kinetics of the ionophore-mediated Ca^{2+} influx and cellular Ca^{2+} -buffering mechanisms; this is likely to be of the order of 1 μM as found with other systems^{12,13}. Alternatively, as Ca^{2+} influx occurs at the plasma membrane and Ca^{2+} sequestration occurs elsewhere (mitochondria, secretory granules), it could be that it is the Ca^{2+} -concentration gradient necessarily set up by the opposing effects of these two processes which acts

as the second messenger in exocytosis. A triggering role for a Ca^{2+} gradient might explain the directionality of exocytosis due to a localised stimulus¹⁴.

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Acetylcholine synthesis by mesencephalic neural crest cells in the process of migration *in vivo*

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Specific to the vertebrate embryo, the neural crest is a transitory structure whose constituent cells migrate extensively through the developing animal and ultimately give rise to many distinct cell types, including the components of the peripheral nervous system^{1,2}. The earliest clear indices of their differentiation have so far been detected only when cells from the crest have reached their destination. This is exemplified by the acquisition of the ability to synthesise and store catecholamines; absent from crest cells before and during their dorso-ventral migration, this ability appears concomitantly with their aggregation into the primary sympathetic ganglia^{3–5}. The chronology of cholinergic maturation, however, is less well defined. Appropriate biochemical markers are demonstrable as soon as parasympathetic or enteric ganglia are formed^{7–9}, but the lack of a suitable cytochemical method is a major obstacle to the identification of any cholinergic cells before then. Although acetylcholinesterase (AChE) is present in migrating neural crest¹⁰, choline acetyltransferase (CAT), the enzyme catalysing acetylcholine (ACh) synthesis, is a much more relevant correlate^{7,11,12}, and definitive evidence for cholinergic differentiation should include the demonstration of ACh-synthesising activity in intact cells^{13,14} or their extracts. We show here that neural crest, as soon as it begins migration, can synthesise ACh.

To overcome the difficulty of applying biochemical techniques to neural crest cells soon after they have begun to migrate, we took advantage of the fact that mesencephalic neural crest in Japanese quail embryos of 8–12 somites is clearly visible underneath the ectoderm as a sheet of cells on either side of the mesencephalic vesicle, and can be dissected out mechanically¹⁵. Each discrete fragment of tissue so obtained is made up of about 1,500 cells and is free from contamination by neural tube or mesenchyme (Fig. 1b). This system provides a unique opportunity for the preparation and study of a homogeneous population of cells actually migrating *in vivo*. Furthermore, it is equally possible to remove the mesencephalic crest at a slightly earlier stage, when it is still in the form of neural folds and

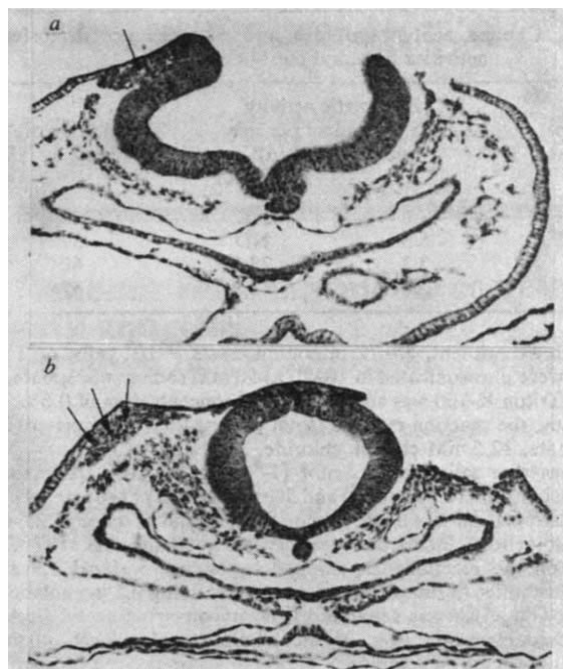


Fig. 1 Removal of the mesencephalic neural crest at the onset of, and during, the migration process in early quail embryos. *a*, Transverse section at the mesencephalic level of a quail embryo of 7 somites stained with haematoxylin. On one side, the migration of crest cells from the neural fold (arrow) is starting. At the opposite side, the crest has been cut out, together with the ectoderm, by means of a microscalpel ($\times 110$). *b*, Transverse section of a 9-somite quail embryo, stained with haematoxylin, showing the mesencephalic neural crest as a thin multi-layered sheet of cells immediately below the ectoderm. Migration is well under way. (Orthotopic grafts of quail neural tube into chick embryos² before the onset of crest migration have shown that the group of cells designated by the arrows on the intact side is made up of a pure population of crest cells.) Again, the contralateral crest has been excised. Note the complete and exclusive removal of the crest cells and the absence of damage to the neural tube ($\times 110$). (In this report, crest obtained from embryos of 5–7 somites is referred to as 'neural fold', that from embryos of 8–12 somites as 'migrating crest'.)

migration is just beginning (Fig. 1a). Each crest fragment at this time contains approximately 1,000 cells.

On incubation with ^3H -choline, isolated migrating mesencephalic neural crest formed a labelled product that co-migrated with ACh on electrophoresis (Fig. 2b). The identity of the radioactive material was confirmed by the demonstration that 85–90% disappeared after AChE treatment (Fig. 2c, d). Crest isolated at the neural fold stage was also able to synthesise ACh, although in significantly smaller amounts (Fig. 2a). Mean values (fmol per 10^4 cells) obtained for ACh synthesis and accumulation during a 4-h incubation were 7.5 ± 2 ($n = 3$) and 25 ± 5 ($n = 5$) for neural fold and migrating crest, respectively; thus, the synthetic ability is about three times greater when the crest has left the neural primordium and is fully engaged in the migration process.

ACh synthesis implies the existence of CAT, and weak but measurable CAT activity was indeed found in homogenates of migrating neural crest (Table 1). However, unequivocal determination of very low CAT levels is difficult if carnitine acetyltransferase (CarnAT) is present. This enzyme is, in fact, quite active in neural crest (Table 1, expts 3, 4). Although acetylation of choline by CarnAT is relatively inefficient^{16,17}, in some circumstances it may contribute significantly to ACh formation *in vitro*^{18,19}. The use of CAT-specific inhibitors²⁰ can reveal such

Table 1 Choline acetyltransferase and carnitine acetyltransferase activities in neural crest homogenates

Expt no.	Enzymatic activity (pmol product per min per mg protein)		% Inhibition of ACh synthesis by NVP
	CAT	CarnAT	
1	3.6	ND	45
2	5.3	ND	57
3	2.2	28.1	60
4	2.8	31.2	52

In each experiment, 68–72 migrating crests ($\sim 10^5$ cells, or 15 μ g protein) were ultrasonicated in 10–20 μ l 50 mM sodium phosphate, pH 7.4, and Triton X-100 was added to a final concentration of 0.5%. For CAT assay, the reaction mixture (total volume 20 μ l) comprised: 5 μ l homogenate, 12.5 mM choline chloride, 300 mM NaCl, 50 mM NaF, 0.1 mM eserine salicylate, 0.1 mM [14 C]acetyl CoA (Amersham; specific activity 58 mCi mmol $^{-1}$) and 50 mM sodium phosphate, pH 7.4. Each determination was made in the presence and absence of 0.5 mM NVP (Calbiochem). Incubations were carried out in the dark at 37 °C for 30 min and the reaction was stopped by adding 5 μ l pH 1.9 electrophoresis buffer (5 times concentrated) containing 0.1 mg unlabelled ACh chloride. ACh was separated from unconverted acetyl CoA by high-voltage electrophoresis of the whole sample (5 kV, 40 min). Radioactivity in the ACh region of the paper was measured as described in Fig. 2 legend. Assay blanks, without enzyme, were run in an identical manner. The electrophoresis step, in addition to permitting the identification of the labelled reaction product, has the advantage of giving low blanks (45–65 c.p.m. including background, corresponding to $\sim 0.03\%$ the initial amount of [14 C]-acetyl CoA); each experimental value was 1.5–3 times higher than the corresponding assay blank. For CarnAT determination, 12.5 mM DL-carnitine replaced choline in the reaction mixture, nonradioactive acetylcarnitine was added at the end of the incubation and the electrophoresis time was increased to 1 h. Results have been corrected for blanks, recovery and counting efficiency. Protein was estimated by the Lowry method, using bovine serum albumin as standard. ND, Not determined.

an artefact and the results obtained with one of them, 4-(1-naphthylvinyl)pyridine (NVP), are included in Table 1. At a concentration that does not affect CarnAT in quail tissue (unpublished results), NVP reduced ACh synthesis by an average of 54%. This is probably an underestimate, as NVP is not a particularly potent inhibitor^{20,21}.

Despite the inference that at least half the ACh produced in cell-free preparations was due to CAT, the involvement of this enzyme in ACh synthesis in whole cells remained to be assessed. Consequently, we examined the effect of saturating concentrations of carnitine on the conversion of 3 H-choline to ACh by intact neural crest cells. If CarnAT was responsible for the synthesis of significant amounts of ACh, labelling of the latter should be reduced in the presence of excess carnitine. Duplicate migrating mesencephalic neural crest preparations were incubated in medium containing both 3 H-choline and 3 H-carnitine (to monitor carnitine uptake and conversion to acetylcarnitine). In one case, a large excess of unlabelled carnitine was also present. The radioactivity accumulated by the cells in choline, carnitine and their acetylated products was subsequently determined in each sample. Table 2 shows the results of an experiment in which the concentration of unlabelled carnitine was more than 1,000 times that of the radioactive precursors. It can easily be calculated that this massive dose increased the intracellular carnitine concentration by a factor of about 500; at the same time, the total radioactivity in acetylcarnitine was reduced by over 75%. In marked contrast, neither the size of the choline pool nor the labelling of ACh was significantly affected. This result clearly shows that choline and carnitine do not compete for the same enzymatic sites in intact cells and strengthens our contention that CAT is responsible for ACh synthesis in neural crest.

We conclude from these studies that mesencephalic crest cells possess a fundamental marker of cholinergic metabolism from the onset of the migratory phase of their development. This may reflect very early differentiation of the fraction of the population that will later give rise to neurones of the ciliary ganglion^{22,23}. On the other hand, ACh-synthesising activity may not necessarily be restricted to presumptive parasympathetic nerve cells. It has been proposed that expression of cholinergic traits is an intrinsic property of differentiating neuroblasts, irrespective of their

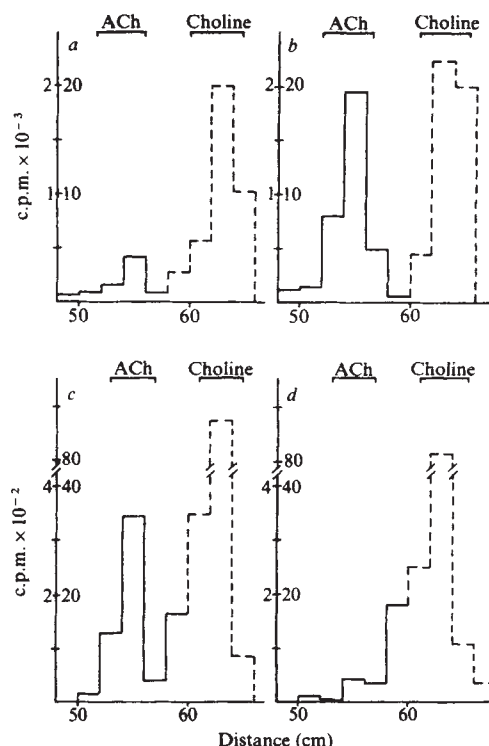


Fig. 2 ACh synthesis by excised mesencephalic crest. The technique was based on that described by Mains and Patterson¹⁴. The incubations were carried out in 200 μ l of medium (L15 lacking choline) containing 15 μ M [methyl- 3 H]/choline (Amersham, 6.4 or 36 Ci mmol $^{-1}$) and 10^{-5} M eserine salicylate. After 4 h at 37 °C in humidified air, the medium was withdrawn, the tissue fragments were washed twice with cold Tyrode's solution and disrupted ultrasonically in 15 μ l of electrophoresis buffer, pH 1.9 (ref. 13), containing unlabelled choline and ACh. The entire sample was applied to Whatman 3 MM paper and electrophoresed at 5 kV for 1.75 h (ref. 13). The positions of ACh and choline were detected with an iodine-based spray²⁵ and sequential, 2-cm-wide rectangles were cut out in the appropriate regions and counted in 10 ml of Beckman 'HP' scintillation fluid after elution/decolouration with 1 ml Na₂S₂O₃ (0.5% w/v in H₂O). Counting efficiency was 45–50%. Background values (35–50 c.p.m.) were obtained from the rectangles immediately preceding the ACh spot. The diagrams show the distribution of radioactivity migrating with ACh (solid lines, outer scale) and choline (broken lines, inner scale), the positions of the marker spots and the total distance of migration towards the cathode. *a, b*, Comparison of ACh synthesis by samples consisting of 20 neural folds (*a*) and 20 migrating crests (*b*). The specific activity of the 3 H-choline was 36 Ci mmol $^{-1}$. The radioactive ACh peak in *b* was not notably altered by prior purification¹⁴ of the precursor, nor by removal of the ectoderm layer associated with the crest. *c, d*, Effects of AChE. The sample consisted of 50 migrating crests. After incorporation of 3 H-choline (6.4 Ci mmol $^{-1}$), the cells were ultrasonicated at pH 4 and centrifuged. The supernatant was adjusted to pH 7 and divided into two equal parts. Each was incubated for 15 min at 25 °C in the absence (*c*) and presence (*d*) of purified *Electrophorus electricus* AChE (Sigma), followed by acidification with concentrated pH 1.9 buffer and electrophoresis. Background counts have been subtracted.

Table 2 Effect of excess carnitine on ACh synthesis

Concentration of unlabelled DL-carnitine	Total net radioactivity (c.p.m.) recovered as:	Acetyl- carnitine	choline	Acetyl- choline
0	1,025	280	8,900	380
40 mM	400	65	8,300	400

Each sample consisted of 20 migrating crests (3×10^4 cells). The first was incubated in medium (L15 without choline) containing 10^{-5} M eserine salicylate, $100 \mu\text{Ci ml}^{-1}$ ($15 \mu\text{M}$) [$\text{Me-}^3\text{H}$]choline and $52 \mu\text{Ci ml}^{-1}$ ($30 \mu\text{M}$) DL[$\text{Me-}^3\text{H}$]carnitine (Amersham). The second sample was incubated in medium containing identical amounts of the radioactive compounds, but to which unlabelled DL-carnitine had been added to a final concentration of 40 mM, thus reducing the specific activity of the precursor from 1.7 Ci mmol^{-1} to $1.3 \text{ mCi mmol}^{-1}$. (Preliminary experiments had shown that extracellular carnitine concentrations of this order were necessary to increase the intracellular level sufficiently to saturate CarnAT.) After incubation for 4 h, the samples were treated as described in Fig. 2 legend, except that unlabelled carnitine and acetylcarnitine markers were added before ultrasonication and the electrophoresis time was increased to 2.25 h. In these conditions, labelled precursors and acetylated products were well separated from one another. The total radioactivity in the regions of the paper corresponding to the substances of interest was determined as indicated in Fig. 2 legend. Background values have been subtracted.

ultimate fate²⁴; it might even be a feature of all neural crest cells (possibly related to the migration process¹⁰). This uncertainty could be resolved directly only by the development of an appropriate, unambiguous cytochemical test. In the meantime, a useful first step towards elucidating the relationship between the early cholinergic phenotype and ontogeny of the peripheral nervous system would be to determine whether crest cells from other axial levels also synthesise ACh and, if so, whether this ability is retained during at least the first stages of sympathetic and sensory ganglion formation.

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Dopamine inhibition of action potentials in a prolactin secreting cell line is modulated by oestrogen

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Secretory activity of the anterior pituitary gland is regulated by the brain through stimulatory and inhibitory substances released from nerve endings in the median eminence of the hypothalamus and carried by the adeno-hypophyseal portal blood system to their respective target cells. These hypothalamic influences are modulated by the feedback action of peripheral hormones. Prolactin (PRL) secreting cells are, at least partially, under the stimulatory influence of thyrotropin releasing hormone (TRH)^{1,2} and of oestrogens³. However, they are mainly controlled by inhibitory substances among which dopamine (DA) is one of the most potent *in vivo* as well as *in vitro*^{4,5}. The inhibitory effect of DA is reversed by oestrogen *in vitro*⁶. The mechanism by which these factors interact on their target cells is poorly understood. The recent discovery that anterior pituitary cells are excitable and that they are able to generate Ca^{2+} -dependent action potentials has led to the suggestion that these effects are involved in a stimulus-secretion coupling at the membrane level⁷⁻⁹. In this paper, we report that DA inhibits both the spontaneous and TRH-induced action potentials in clonal PRL pituitary cells. In addition, oestradiol-17 β is able to reverse the inhibitory effect of DA.

In this study GH3 B6 cells, a subclone of GH3 rat prolactin cell line were used. These cells secrete prolactin and respond to both TRH and oestradiol². Stable intracellular recordings lasting between 10 min and 1 hour were obtained from 260 cells. Of the cells tested 51% were electrically excitable and displayed action potentials when depolarised by an outward current. Twenty-six per cent of the cells were spontaneously active. These action potentials were mainly Ca^{2+} -dependent (Fig. 1a).

Sixty-two spontaneously firing cells were tested for an effect of DA; in 25 cells (40%), DA injected close to the cell provoked an inhibition of firing within 30–60 s (Fig. 1c). This inhibitory effect was preceded by a rapid decrease of the input resistance with no detectable change in the resting membrane polarisation (Fig. 1b); in 37 cells DA had no effect. In addition, DA inhibited

Table 1 Effect of ^3H -DA on PRL release and ^3H -DA binding in GH3 B6 cells

	Control	^3H -DA	^3H -DA + DA
PRL release (ng per mg cell protein)	283 ± 18.2	193 ± 19.8*	205 ± 6.9*
^3H -DA bound (d.p.m. per mg cell protein)	—	2,380 ± 196	676 ± 48

200,000 cells per 2-ml dish were grown for 5 days as previously described¹⁰. Before the experiment the cells were rinsed with warm Ham F10+HEPES 20 mM, pH 7.2 (F10-H). They were thereafter incubated in 1 ml of fresh F10-H with or without the addition of ^3H -DA (16 pmol ml, 6.6 Ci mmol⁻¹) and unlabelled DA (10 nmol ml⁻¹) for 30 min at 37°C in 95%–5% air–CO₂ humidified incubator. The incubation was stopped on ice. The cells were collected for protein assay and liquid scintillation measurement of bound radioactivity¹⁰. The half life of DA in such conditions was 30 min as measured in parallel dishes by J. P. Tassin (U. 114, INSERM, Collège de France) using a specific enzymatic radioassay. The medium was collected for PRL radioimmunoassay which was expressed in nanogram equivalent of rat standard RP-1 from the NIAMDD kit. Each point is the mean of triplicate ± s.e.m.

* $P < 0.05$.

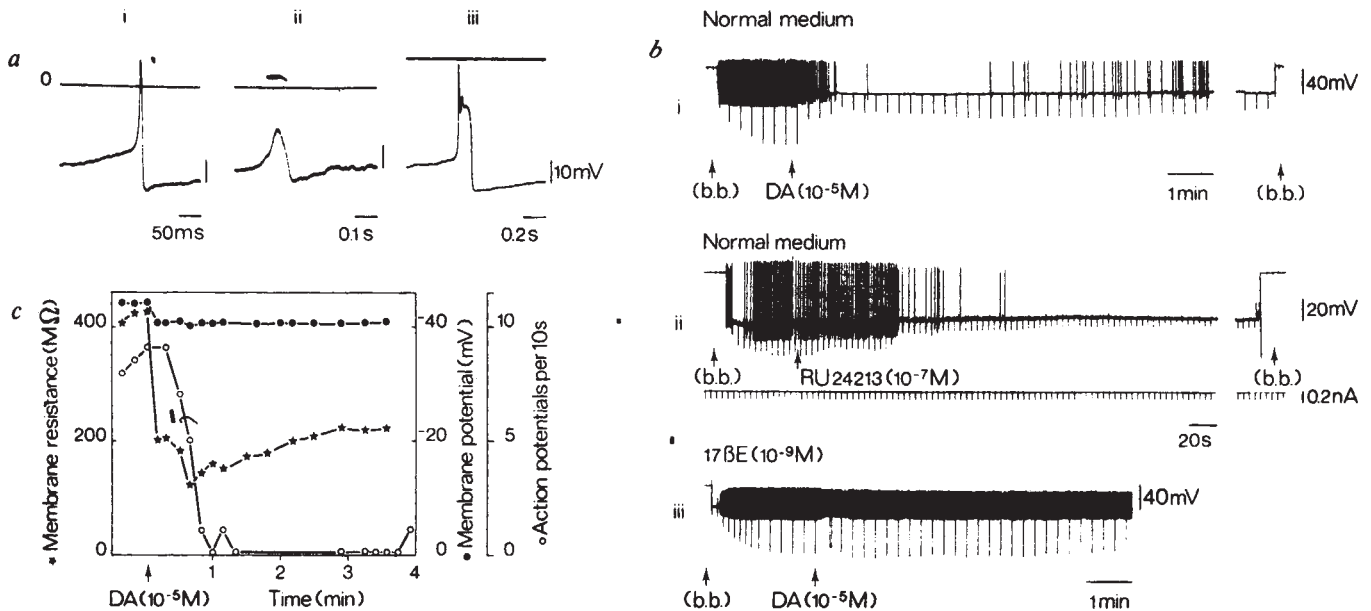


Fig. 1 Effect of DA on the firing of action potentials in the GH3 B6 prolactin secreting cell line. The cells were grown in Ham's F10 solution supplemented with 15% horse serum and 2.5% fetal calf serum. Cells were cultured for 5 to 7 days before being used in electrophysiological studies. The experiments were carried out in a temperature controlled room ($26 \pm 1^\circ\text{C}$). Ten minutes before recording, the culture medium was replaced by a bathing solution with the following composition (in mM): NaCl 142.6; KCl 5.6; CaCl_2 10; glucose 5; HEPES buffer 5 (pH 7.4). Stock solutions of oestradiol-17 β (17 β -E) were prepared in ethanol and diluted in the above solution; the final ethanol concentration being 0.01%; TRH (Beckmann) and DA (Hoffman-La Roche) were dissolved in bathing solution. The substances to be tested were delivered via a pneumatic ejection system which allowed rapid administration (0.5 s) of very small volume (1–5 nl) of solution from micropipettes positioned close to the membrane of the cell tested¹³. Twenty-six per cent of the cells ($n = 260$) were spontaneously active.

a, The all-or-none action potential had a positive overshoot and a prominent after-potential (i); the action potentials were reduced following the application of D 600, a known blocker of Ca^{2+} channels (ii); action potentials were still encountered in medium in which sodium chloride was replaced by choline chloride, however their size and shape were changed and no overshoot was observed (iii); the zero membrane potential is indicated by 0. **b**, The effect of DA on spontaneous action potentials. Vertical bars represent a measure of the membrane input resistance obtained by injecting a current of 0.16 nA with a bridge amplifier. i, Effect of DA on action potential firing and membrane resistance in an oestrogen depleted medium. ii, Effect of the DA agonist RU 24213 (Roussel-Uclaf) on action potential firing and membrane resistance in an oestrogen depleted medium. The current used to monitor the cell membrane resistance is shown at the bottom of the figure. In (i) and (ii) the bridge balance is indicated (b.b.). iii, Effect of DA when oestradiol-17 β (10^{-9} M) was added to the recording medium. **c**, Time course of changes in membrane electrical properties following administration of DA (10^{-5} M) on the cell represented in b(i). $\star$, Membrane resistance; $\circ$, frequency of firing of action potentials; $\bullet$, membrane potential.

the TRH-induced action potentials. The DA agonist RU24213 induced a response comparable to that of DA (Fig. 1b). In the same way as for DA, the inhibitory effect of RU24213 on action potential firing was always associated with a significant decrease in membrane resistance. Conversely, the DA antagonists haloperidol (10^{-6} M) and chlorpromazine (10^{-6} M) suppressed the inhibitory effect of DA on action potentials when they were administered to a spontaneously firing cell (Fig. 2a). Surprisingly, an increase in conductance occurred without any noticeable change in membrane potential. The spontaneously firing cells recorded in this study have a resting membrane potential of -45 ± 3 mV which is not near the normal equilibrium potential of any ions likely to be involved in the DA effect. The ionic nature of the currents involved in the DA effect has not yet been investigated. However, it is possible that DA increases the Cl permeability of the cell; a leakage of KCl from the tip of the KCl electrode may elevate the internal Cl concentration and bring the equilibrium potential for this ion close to -45 mV. Several arguments support this hypothesis: for example, when the resting membrane potential was displaced towards -80 mV, it was difficult to maintain a steady potential, although DA was able to induce a long-lasting depolarisation together with an increase in membrane conductance.

The DA inhibition of both spontaneous and TRH-induced action potentials may be related to the effect of DA on PRL secretion. A significant decrease of PRL release in GH3 B6 cells was induced within 30 min of exposure to ^3H -labelled DA (16 nM) (Table 1). Simultaneously the cells showed a binding capacity for ^3H -DA and this was partially inhibited by an excess of unlabelled DA (Table 1). The inhibitory effect of DA on action potential firing and the decrease in PRL secretion follow-

ing exposure to DA are consistent with the stimulus-secretion coupling hypothesis. However, these two effects cannot be compared in a quantitative manner, as the method of DA application in the two experiments is different. Also, we do not know the exact amount of DA which reaches the cell membrane when DA is administered by pressure injection. We have previously shown that the DA agonist CB 154 decreased TRH-induced PRL release in GH3 B6 cells without modifying the binding of ^3H -TRH (ref. 10). Although these cells are of neoplastic origin, they exhibit a binding capacity and a response to DA similar to that of normal anterior pituitary cells⁵. The fact

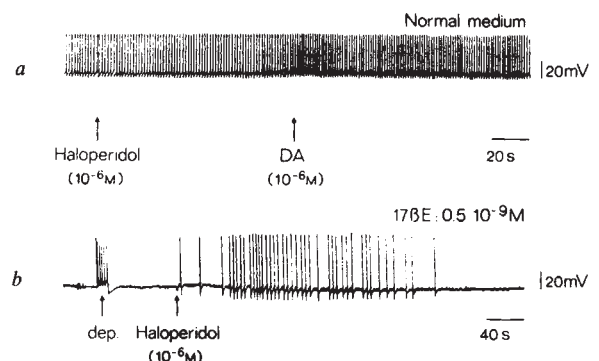


Fig. 2 Effect of haloperidol on action potential firing in GH3 B6 cells. **a**, Haloperidol (10^{-6} M) was administered to a spontaneously firing cell and antagonised the inhibitory effect of DA. **b**, Haloperidol-induced action potentials in a silent but excitable cell when oestradiol-17 β (17 β -E) was present in the bathing medium. The excitability of the cell was tested by a depolarisation of 0.16 nA.

that DA inhibits both the firing and hormone release supports the hypothesis that Ca^{2+} -dependent action potentials are involved in the release of anterior pituitary hormone^{7,8}. The mechanism by which DA inhibits the firing of PRL cells is not clearly understood. From our experiments this mechanism appears to be different from that described in central neurones¹¹, in that no change in the membrane polarisation was observed. Previously, we demonstrated that TRH, which stimulates the secretion of PRL, elicits action potential firing by increasing the membrane resistance without any noticeable depolarisation. These data are in agreement with the finding of Martin *et al.*¹², although Ca^{2+} ions are necessary for the secretion of pituitary hormones, depolarisation is not tightly linked to the release process. Thus, in our experimental conditions, DA appears to act on membrane excitability and resistance of GH3 B6 cells in a way opposite to TRH, although the exact mechanism involved is not known.

The percentage of cells inhibited by DA was dependent on the level of oestrogen in the growth medium. When the cells were grown for at least 24 h in an oestrogen-depleted medium, the percentage of DA-inhibited cells reached 80% (43 out of 54), compared to 40% in normal medium. When oestradiol-17 β (10^{-9} M) was added to the recording solution, in all cells tested, DA had no effect on the action potential firing or input resistance ($n=30$) (Fig. 1c). We have previously shown that oestradiol-17 β when directly applied to the cell, provokes action potential firing⁹. DA did not inhibit this oestrogen-induced electrical activity. Moreover, haloperidol had a slight effect when added alone, and elicited a short burst of action potentials when oestradiol-17 β was present in the recording solution (Fig. 2b); haloperidol was ineffective in an oestrogen-free solution. These results are in agreement with the recent observations by Raymond *et al.*⁶ that oestrogen has a potent antidopaminergic effect on prolactin release in primary cultures of anterior pituitary cells. Furthermore, it has been recently shown that oestrogen interacts with anterior pituitary DA receptors¹⁴. The inhibitory effect of DA on action potential firing does not seem to be mediated by a change in membrane polarisation, and so differs from the mechanisms of action of DA so far described¹¹. However, at least two classes of DA receptors have been described¹⁵. The first class of receptors, which increase cyclic AMP synthesis, were found in the caudate nucleus. The second class, which do not increase cyclic AMP synthesis, were described in nigro-neostriatal neurones and mammothroph cells of the pituitary¹⁵. Recently, Euvrard *et al.*¹⁶ have shown that oestrogen antagonises the inhibitory responses of DA in the striatum. The GH3 B6 cell line may provide a model for the study of some postsynaptic dopaminergic systems, which are of particular interest when considering the role of dopaminergic systems in the modulation of mood and behaviour. Moreover, the model could be useful in the study of the interactions of oestrogen and DA in the control of PRL release at the pituitary level and more so, the action of oestrogen on brain function.

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Benzodiazepine and β -adrenergic receptor ligands independently stimulate phospholipid methylation

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The stepwise methylation of phosphatidylethanolamine by two methyltransferase synthesises phosphatidylcholine in membranes¹. Although the CDP-choline pathway is the major route for phosphatidylcholine synthesis, recent work in our laboratory has found that the methylation pathway is metabolically highly active. The phospholipid methyltransferases, their substrates and their products are asymmetrically distributed in the cell membrane. Methyltransferase I, located on the cytoplasmic side of the membrane, transfers a methyl group from S-adenosylmethionine to phosphatidylethanolamine to form phosphatidyl-N-monomethylethanolamine. Methyltransferase II, on the exterior surface of the bilayer, catalyses two additional N-methylations to form phosphatidylcholine. The asymmetric distribution of methyltransferases results in the synthesis and translocation of phosphatidylcholine to the exterior bilayer surface². The synthesis and translocation of methylated phospholipid affects many membrane events, such as fluidity³, coupling of the β -adrenergic receptor with adenylate cyclase⁴, the number of β -adrenergic receptors⁵, Ca^{2+} -ATPase activity⁶, leukocyte chemotaxis⁷, mast cell histamine secretion⁸ and lymphocyte mitogenesis⁹. We now report that stimulation of a benzodiazepine receptor with anti-anxiety benzodiazepines, and stimulation of the β -adrenergic receptor with β -agonists, increase phospholipid methylation in C₆ astrocytoma cells. Furthermore, simultaneous addition of benzodiazepine and β -adrenergic agonists increases methylation in an additive manner.

Cultured C₆ astrocytoma cells have both β -adrenergic receptors and 'peripheral-type' benzodiazepine receptors¹⁰. The C₆ astrocytoma avidly binds ³H-diazepam ($K_D \approx 17$ nM) at a single and saturable class of binding sites. The 'peripheral-type' benzodiazepine receptor of C₆ astrocytoma is differentiated from the 'central-type' benzodiazepine receptor found in the cortex by their differing affinity for two ligands, Ro-5-4864 (a 4-chloro derivative of diazepam) and clonazepam. Ro-5-4864 is potent in displacing ³H-diazepam from the peripheral type site ($K_i = 2.3$ nM), but is ineffective in the 'central-type' site. Clonazepam is ineffective in displacing ³H-diazepam from the 'peripheral-type' site ($K_i = 2,000$ nM) whereas it is very potent in the 'central-type' receptor ($K_i = 0.3$ nM). The 'central-type' receptor has been extensively characterised by binding studies¹¹, biochemical studies¹², electrophysiological studies¹³ and interactions with the GABA receptor for γ -aminobutyric acid¹⁴. The 'peripheral-type' benzodiazepine receptor has been characterised only by ³H-diazepam binding studies.

The effect of benzodiazepines on phospholipid methylation was examined by incubating C₆ astrocytoma cells with [³H]methionine. The ³H-methyl-labelled phospholipids were examined by extraction in chloroform/methanol⁴. Separation of ³H-methylated products by TLC demonstrated that 70% of ³H-methyl groups were incorporated into phosphatidylcholine, 5% into phosphatidyl-N-dimethylethanolamine and 15% into phosphatidyl-N-monomethyl-

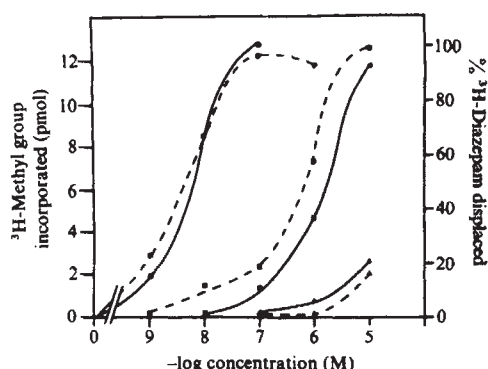


Fig. 1 Benzodiazepine receptor ligands, phospholipid methylation and displacement of ^3H -diazepam from the receptor binding site. C_6 astrocytoma cells (41–68 passages) were grown for 4–5 d until confluent in Dulbecco minimal essential media with 4.5 g l^{-1} glucose, 5% fetal calf serum and 4 mM L-glutamine, in FB6-TC multi-dish trays (Linbro) for phospholipid methylation assays (0.5–0.8 mg protein per well), and in Falcon flasks for ^3H -diazepam binding assays. The incorporation of ^3H -methyl groups into phospholipids was studied by aspirating the media from each well of the multi-dish tray, and adding 1 ml of Media 199 (Microbiological Associates), containing $2 \times 10^{-4} \text{ M}$ ($\pm$)methionine, and 30 μCi [^3H]methionine (1 mCi ml^{-1} , NEN) per well. The cells were incubated for 25 min to equilibrate the isotope, and various concentrations of ligand were then added to the wells. After a 30-min incubation, the wells were washed twice with iced 0.9% NaCl. 10% Trichloroacetic acid (TCA), 1 ml, was added, the wells were scraped with a rubber policeman, and the cells transferred by pipette to test tubes. After centrifugation at 27,000g for 10 min the supernatant was discarded. Phospholipids were extracted from the TCA precipitate with chloroform/methanol, followed by two washes with 0.1 M KCl in 50% methanol, as previously described¹. The organic phase, 1 ml, was evaporated in a scintillation vial, resuspended in scintillation fluid, and counted in a spectrometer. The results are expressed as pmol ^3H -methyl groups incorporated per 30-min incubation per well, and are the difference in ^3H -methyl incorporation between ligand-stimulated and control cells, in at least three separate experiments. Ligands were Ro-5-4864 ($\bullet$), clonazepam ($\blacksquare$) and chlordiazepoxide ($\blacktriangle$). After the preincubation, 16.4 ± 4.2 pmol (s.e.m.) of methyl group were found in the phospholipids per mg protein. During the incubation in the absence of ligand for a further 30 min, 24.2 ± 2.1 pmol of methyl group were incorporated per mg protein. Variations occur in different batches of astrocytoma cell cultures, but within the same experiment, were approximately $\pm 5\%$. ^3H -Diazepam binding was measured in membranes prepared from cells. Cells were washed twice with phosphate-buffered saline, pH 7.4, and scraped into 10 mM Tris HCl, pH 7.0, at 25°C, homogenised by a Polytron homogeniser, and centrifuged at 40,000g for 10 min. The pellet was resuspended in Tris-buffer to a concentration of 2–3 mg protein ml^{-1} . The binding assay was carried out in a final volume of 500 μl , with 100 μl membrane protein, 50 μl of various competing ligands, 4 mM ^3H -diazepam (80 Ci mmol^{-1} , NEN) and incubated for 30 min at 4°C. The reaction was stopped by adding 7 ml iced Tris-buffer and the solution rapidly filtered through a GF/B filter (Whatman). The filter was washed twice with 7 ml iced Tris-buffer, then counted in a liquid scintillation spectrometer. Specific binding of ^3H -diazepam is the difference between total binding and nonspecific binding in the presence of 10^{-5} M Ro-5-4864. In a typical experiment, 700 fmol of ^3H -diazepam was bound per mg protein and specific binding was 95% of the total binding. Data are expressed as the % displacement of specifically bound ^3H -diazepam, and are the mean of duplicate determinations. Ligands tested were Ro-5-4864 ($\bullet$), clonazepam ($\blacksquare$) and chlordiazepoxide ($\blacktriangle$).

ethanolamine. Following preincubation of cells with [^3H]methionine, incubation with benzodiazepine derivatives increased ^3H -methyl incorporation into phospholipids (Fig. 1). Methylation was increased by these derivatives in a dose-dependent manner. To determine whether the astrocytoma cells contain the 'central-type' or 'peripheral-type' benzodiazepine receptor, three derivatives of benzodiazepine were used. Their relative potency in increasing phospholipid was characteristic of the 'peripheral-type' receptor (Ro-5-4864 > clonazepam > chlordiazepoxide). Furthermore, the potency of several benzodiazepines in stimulating methylation closely matched their potency in displacing ^3H -diazepam from the receptor binding site (Fig. 1).

Previous work with rat reticulocytes⁴ and HeLa cells¹⁵ demonstrated that stimulation of the β -adrenergic receptor increased the methylation of membrane phospholipids. Similarly, in astrocytoma cells, the addition of the β -adrenergic ligands increased phospholipid methylation. When astrocytoma

cells preincubated with [^3H]methionine were incubated with β -adrenergic agonists, the appearance of methylated phospholipids increased in a dose-dependent fashion. The capacity of these agonists to increase methylation matched for the most part their ability to stimulate adenylate cyclase (Fig. 2). A greater degree of stereospecificity was shown with phospholipid methylation than with adenylate cyclase. Their order of potency in increasing cyclic AMP levels, and in displacing ^3H -dihydroalprenolol from the receptor binding site (not shown) was typical for a β_1 -adrenergic receptor (isoprenaline > noradrenaline = adrenaline) and exhibited marked stereospecificity ((-)-isoprenaline >> (+)-isoprenaline).

As both the benzodiazepines and the β -adrenergic agonists increase phospholipid methylation, they could be acting on the same methyltransferases or on separate domains associated with the specific receptors. If the β -adrenergic receptor and benzodiazepine receptor increased phospholipid methylation on the same enzyme, stimulating both receptors simultaneously would

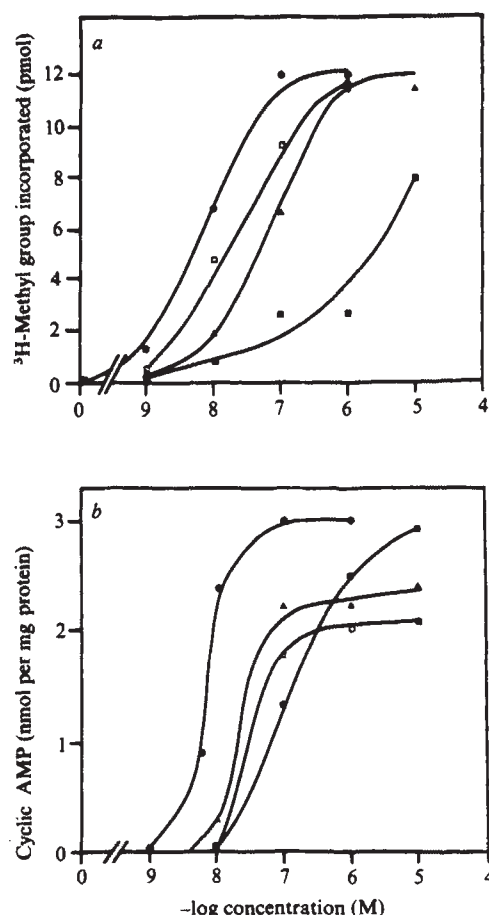


Fig. 2 β -Adrenergic receptor agonists, ^3H -methyl groups incorporation into phospholipids, and cyclic AMP levels. **a**, Stimulation of phospholipid by β -adrenergic agonists. C_6 astrocytoma cells were grown in multi-dish-trays as previously described. Cells were incubated for 25 min with [^3H]methionine, and then incubated with various concentrations of agonists for 30 min. The cells were precipitated with TCA and the phospholipids extracted and assayed for ^3H -methyl incorporation as described in Fig. 1 legend. The results are expressed as pmol of ^3H -methyl groups incorporated per 30-min incubation per well, and are the difference in ^3H -methyl incorporation between agonist stimulated and control cells in at least three experiments. Agonists used were (-)-isoprenaline ($\bullet$), (-)-adrenaline ($\square$), (-)-noradrenaline ($\blacktriangle$) and (+)-isoprenaline ($\blacksquare$). **b**, Increased cyclic AMP levels following β -adrenergic receptor stimulation. Intracellular cyclic AMP levels were measured by incubating cells in multi-dish trays for 10 min in various concentrations of agonists. The incubation was stopped by aspirating the media and adding 1 ml of 0.1 M HCl. The HCl aliquot was then lyophilised and redissolved in water. The cyclic AMP content was determined by a protein binding method¹⁷. The data are expressed as nmol cyclic AMP per mg protein per 10-min incubation, and are the mean of duplicate determinations in two experiments. Ligands tested, and their symbols, are the same as in **a**.

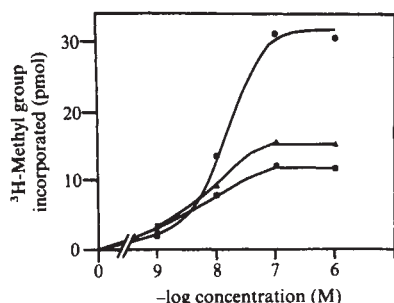


Fig. 3 Effect of simultaneous stimulation of benzodiazepine and β -adrenergic receptors on phospholipid methylation. Following preincubation with [3 H]methionine, as described in Fig. 1 legend, astrocytoma cells were incubated for 30 min with increasing concentrations of Ro-5-4864 ($\blacktriangle$), (—)isoprenaline ($\blacksquare$) or both drugs ($\bullet$). The incorporation of 3 H-methyl groups into phospholipids was assayed as described in Fig. 1 legend. The data are the mean of duplicate determinations of three experiments.

increase methylation no more than if either receptor were stimulated maximally. To test this possibility, the cells were treated with increasing concentrations of Ro-5-4864 or isoprenaline, or both drugs together (Fig. 3). Simultaneous stimulation of both benzodiazepine and β -adrenergic receptors increased the appearance of methylated phospholipids in an additive fashion. These results suggest that the methyltransferases are located in separate domains in the vicinity of the respective receptors. Each domain is independently affected by receptor ligands.

Previous work has demonstrated that binding of the β -adrenergic receptor by its agonists increases the methylation of membrane phospholipids¹. The synthesis of phosphatidyl-N-monomethylethanolamine by methyltransferase I increases membrane fluidity³ and enhances coupling between the β -adrenergic receptor and adenylate cyclase⁴, presumably by facilitating lateral mobility of the receptor. The same effects may occur in astrocytoma cells, where β -adrenergic receptor stimulation also increases phospholipid methylation. The activities of both methyltransferases I and II are stimulated by either isoprenaline or benzodiazepines.

Little is known about the physiological or biochemical changes resulting from stimulation of the 'peripheral-type' benzodiazepine receptor, or whether the differences between the 'central' and 'peripheral' receptor reflect an intrinsic difference between these sites. Alternatively, the membrane environment of the receptor may modify its pharmacological properties¹⁶. The demonstration that benzodiazepine derivatives enhance phospholipid methylation is the first known biochemical event related to this receptor. As phospholipid methylation alters a variety of membrane functions, this observation may provide a rationale for additional studies of whether these 'peripheral-type' receptors have specific functions reported for the 'central-type' receptor.

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How the size of motoneurons determines their susceptibility to discharge

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The susceptibility of motoneurons to discharge by physiological inputs varies inversely with their size^{1,2}. This relationship has far-reaching consequences for the control of muscular tension, and also accounts for significant differences in the properties of muscle fibres and motor units^{3–5}. The probable basis for the relationship is that the amplitude of excitatory postsynaptic potentials (e.p.s.ps) is also correlated inversely with cell size^{6,7}, but no satisfactory explanation for this finding is known. We now describe experiments which indicate that afferent impulses invade the relatively simple ramifications of fibres going to small motoneurons more completely than the extensive arborisations going to large cells. This results in activation of a higher percentage of the synaptic endings on small cells, which produces larger e.p.s.ps in them and accounts for their greater susceptibility to discharge.

Experiments were designed to test the following hypothesis. As motoneurons of different sizes reportedly have equal densities of synaptic boutons⁸, those with large surface areas must have proportionally more boutons. In particular, if large motoneurons supplying the medial gastrocnemius have more endings from Ia fibres coming from that muscle, this is due, not to the fact that they receive projections from more Ia fibres, but to the more extensive arborisation of individual Ia fibres on these cells, because each motoneurone receives terminals from essentially all Ia fibres coming from its muscle⁹. The more numerous branch points, terminal boutons and *boutons en passant* in extensive arborisations presumably lower the safety factor for propagation¹⁰. Experimental evidence indicates that

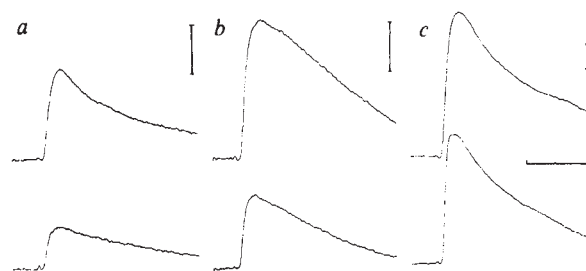


Fig. 1 Examples of aggregate e.p.s.ps recorded from three medial gastrocnemius motoneurons to illustrate the effects of post-tetanic potentiation on cells of different size. Microelectrodes with resistances of 3–10 M Ω were used to record intracellular potentials of motoneurons in cats deeply anaesthetised with sodium pentobarbital. After locating intraspinal regions where antidromic volleys in the axons of MG motoneurons evoked large electrical 'fields', the ventral roots were severed to eliminate motoneurone backfiring which interferes with the recording of their e.p.s.ps. The input resistance of each penetrated cell was obtained by measuring the effects of hyperpolarising and depolarising currents on the amplitudes of antidromic spikes (spike height method)¹⁶. Eighteen measurements at nine levels of current injection were made. Only cells yielding linear plots of spike height against current were accepted for definitive study. Each of the lower records is the average of 16 monosynaptic, aggregate e.p.s.ps elicited by brief 1 s⁻¹ shocks supramaximal for all Ia fibres in the gastrocnemius nerve. After these control responses had been obtained, the muscle nerve was stimulated at 500 s⁻¹ for 10 s and e.p.s.ps were again recorded at 1 s⁻¹. The averages of the 3rd to the 18th e.p.s.p. after these tetani are shown in the upper traces. Increases in the size of these e.p.s.ps are of presynaptic origin, being associated with hyperpolarisation of the Ia fibres following a tetanus¹⁵. The present study suggests that hyperpolarisation enhances local current flow during action potentials, facilitating invasion of terminal arborisations and activating more synaptic knobs. Vertical scale bars, 2.0 mV; horizontal scale bar, 5.0 ms; IR values: a, 0.3 M Ω ; b, 1.1 M Ω ; c, 3.0 M Ω .

an impulse in a motor axon may fail to invade all its branches¹¹ and that transmission failures may occur in Ia synapses on motoneurons¹². It follows that the larger a motoneuron is, the more extensive are the terminal arborisations on it. Therefore, the possibility that impulses in them will die out at certain points will be correspondingly greater and the percentage of Ia endings that are activated by maximal volleys in Ia fibres will be smaller. Anything that facilitates invasion of Ia terminals and activation of synaptic endings should lead to larger aggregate e.p.s.ps and this tendency should be greater in large than small motoneurons.

To test this hypothesis, intracellular recordings were made from motoneurons of anaesthetised cats. First, the input resistance (*IR*) of each motoneuron was measured to provide a reliable estimate of its size^{13,14}. Then, the medial gastrocnemius nerve was stimulated at 1-s intervals with brief shocks that set up afferent volleys in all its Ia fibres. The monosynaptic e.p.s.ps evoked by them were recorded and a series of 16 of these responses were averaged electronically and written out on an x-y plotter. The muscle nerve was then tetanised at 500 s⁻¹ for 10 s and a post-tetanic series of e.p.s.ps was recorded and averaged. Lloyd¹⁵ showed that conditioning tetani of this frequency and duration caused hyperpolarisation of the Ia fibres and a dramatic increase in the amplitude of the monosynaptic reflexes elicited by volleys in them. This 'post-tetanic potentiation' (p.t.p.) fell gradually to control levels within 2-3 min.

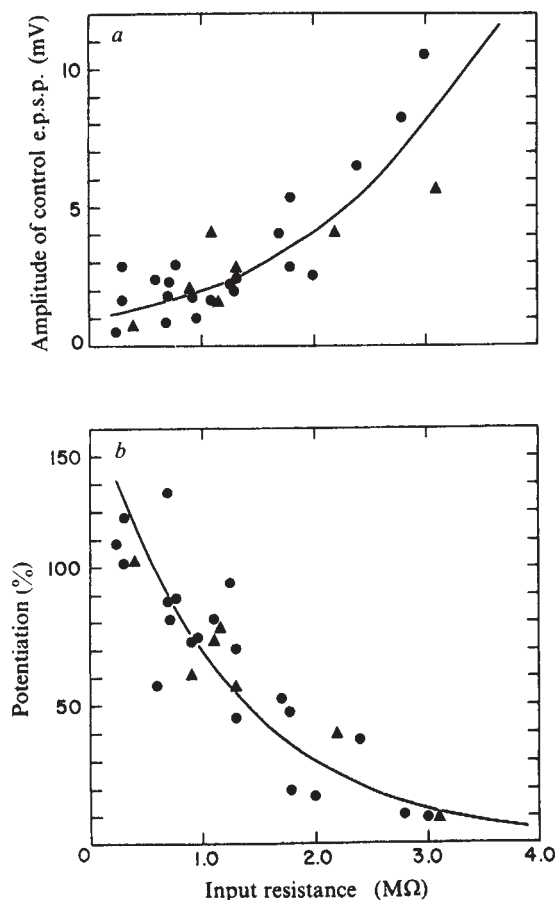


Fig. 2 Relationship between input resistances of motoneurons (in MΩ) and amplitudes of their aggregate e.p.s.ps (in mV) before (a) and during (b) post-tetanic potentiation. Data were pooled from nine experiments. Measurements from a few semitendinosus motoneurons (▲) are plotted with those from medial gastrocnemius (●) to illustrate the essential similarity of the relationship for both species of motoneuron. Comparison of a and b indicates that motoneurons with the largest control e.p.s.ps exhibited the least potentiation and those with the smallest control e.p.s.ps were potentiated the most, as our hypothesis predicts. The exponential curves through the two sets of data points were plotted by the method of least squares.

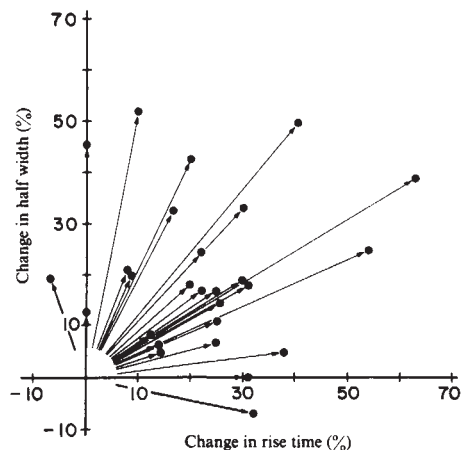


Fig. 3 Plot of changes in rise times of e.p.s.ps against changes in halfwidths following a tetanus to the muscle nerve at 500 s⁻¹ for 10 s. Arrows indicate directions of change. The time courses of e.p.s.ps in these experiments were sometimes altered by afferent impulses in group II fibres, which also project monosynaptically to motoneurons¹⁷. The data plotted here were obtained from records where there was no evidence of group II inputs, but the possibility of a contribution from them could not be eliminated entirely.

Figure 1 shows typical effects of p.t.p. on the e.p.s.ps of three motoneurons of different sizes. The lower traces in the three pairs of records illustrate that the average size of the aggregate e.p.s.ps before potentiation varied directly with the *IR* of the motoneuron (see values in legend to Fig. 1). This confirmed previous observations⁷. The upper traces are the averages of 16 e.p.s.ps following the tetanus. The per cent potentiations in a, b and c were 123, 92 and 11, respectively.

Figure 2a plots the relationship between input resistance and amplitude of control e.p.s.ps in data pooled from nine experiments. Figure 2b illustrates the way in which per cent potentiation was related to input resistance in the same experiments. As our hypothesis predicts, the greatest potentiation occurred in motoneurons with the lowest *IR*, that is, in the largest cells. The least potentiation and the largest control e.p.s.ps were found in motoneurons with the highest *IR* values. Less branching would be expected in Ia fibres approaching these small cells and few inactive endings should be available to increase synaptic input during p.t.p. It has generally been assumed that p.t.p. is due to release of more transmitter from a fixed number of active endings. This assumption, however, would predict that the effect of p.t.p. is independent of cell size and is no longer tenable.

If our hypothesis is correct, p.t.p. might be expected to cause small increases in the conduction time required for Ia volleys to invade additional terminals and to activate more synaptic knobs. The flow of synaptic currents at the various endings might be less synchronous, that is, more prolonged due to these delays¹⁰, but it is difficult to predict the effects of p.t.p. on the time course of e.p.s.ps without precise information about the geometry of the terminal arborisation of Ia fibres. Figure 3 plots the changes in rise time in e.p.s.ps against the changes in their halfwidths during p.t.p. In general, rise times and halfwidths increased during p.t.p. as predicted, but the magnitudes of these changes do not seem to be related (see Fig. 3 legend).

These experimental findings are consistent in all respects with our proposed hypothesis. They suggest that invasion of Ia terminals is a graded process that is generally more complete in the terminal arborisations on small cells because they have fewer branch points. The percentage of Ia boutons that are activated by afferent impulses depends on this process. The surface area that a motoneuron presents to approaching Ia fibres may influence their tendency to branch and, thus, indirectly determine its own susceptibility to discharge.

Activation of pre-existing but inactive synaptic endings, which our findings imply, provides the functional equivalent of

new connections. Experiments to determine whether a similar mechanism, with a more prolonged time course, is used in the circuits involved in memory and learning might offer promising leads.

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Single ionic channels observed in tissue-cultured muscle

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The extracellular patch clamp technique developed by Neher *et al.*¹ to record the responses of single channels in skeletal muscle has provided firm evidence for the two-state nature of the conductance event in nicotinic endplate channels. We report here the use of the extracellular patch technique to record single-channel responses from tissue-cultured chick skeletal muscle cells. The temperature dependence of channel conductance and gating kinetics shows no evidence of discontinuous behaviour between 17 and 37 °C.

The ability to record single-channel responses makes it possible to investigate properties of excitable cells which are otherwise inaccessible. Fischbach and Lass², have reported that in a tissue-cultured preparation of chick 'myoballs', the single-channel conductance (as estimated by fluctuation methods) increased with temperature to about 20 °C, and then remained constant at about 30 pS. The channel open time, as estimated by the half-power frequency of the fluctuation power spectrum, did not show any abrupt change with temperature. Their data present two simple alternative interpretations: (1) that there are two or more populations of channels, high conductance and low conductance, which change proportions as a function of temperature; or (2) that the whole population of channels shows identical, discontinuous, responses to temperature. Using kinetic techniques, the first alternative cannot be distinguished from the second as the populations are apparently kinetically indistinguishable. However, the problem could be solved if the response of the individual events could be resolved.

Using 100 nM suberyldicholine as an agonist, we measured single-channel responses in cells at rest and in cells in micro-electrode voltage clamp conditions. Suberyldicholine was chosen as the agonist as its channel open time is longer than that for other agonists. A typical record of channels observed in an unclamped cell is shown in Fig. 1. The salient features of the record are similar to the results reported for frog muscle^{1,3}. Two-state current pulses of inward current, shown as downward deflections, are the most striking features. We calculated the single-channel conductance assuming a reversal potential of

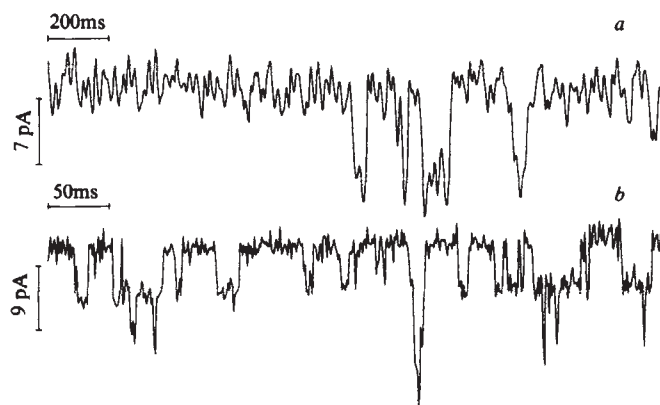


Fig. 1 Digitised membrane current traces showing pulse-like agonist-activated current flow through single channels. Downward deflections indicate inward current. Both records were filtered at a bandwidth of 280 Hz. *a*, 23.5 °C, membrane potential –49 mV, at rest. *b*, Different cell, 38 °C, membrane potential –102 mV, under voltage clamp. Cultures were prepared from 10–12-day-old Leghorn embryos using mechanical disaggregation in divalent ion-free phosphate-buffered saline⁴. The cells were plated on gelatin coated plastic tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% heat inactivated horse serum and 2% embryo extract. The cells were treated with 10 μ M cytosine arabinoside to prevent overgrowth of fibroblasts¹⁴. For experiments, the dishes were filled with HEPES-buffered Hank's saline containing 10% heat inactivated horse serum covered with a layer of mineral oil, and placed on the temperature-controlled stage of an inverted, phase contrast microscope. Single-channel records were made either on cells at rest with an intracellular micro-electrode to record potential or in voltage clamp conditions using a standard two microelectrode voltage clamp. Extracellular patch recording pipettes were made according to techniques described by Neher *et al.*¹. The pipettes were filled with Hanks' saline containing 50–100 nM suberyldicholine. Pipette resistances varied from 1–5 M Ω in solution, increasing to 3–20 M Ω when placed against the cell surface. The patch recording system was essentially the same as described previously¹ with the addition of an automatic drift compensation circuit (E. Neher, unpublished) and an internal calibration circuit. Data were recorded on analogue magnetic tape for later analysis.

0 mV and a linear current voltage relationship^{4,5}. At 22 °C we calculated a mean channel conductance of 60 pS (compared with 48 pS observed by Jackson and Lecar⁵). Working with frog extrajunctional receptors, Neher and Sakmann⁶ estimated the single-channel conductance to be about 15 pS at 8 °C. Even if a Q_{10} of 1.5 is assumed, the conductance is significantly lower than the values reported here for homeotherms. In adult rat, Sakmann⁷ has reported a channel conductance at 22 °C of 34 pS at the endplate, and 25 pS extrajunctionally. The tissue-cultured preparations seem to have a larger channel conductance than the adult tissues.

The open channel duration is an exponentially distributed random variable (Fig. 2). The mean channel open time was estimated as the time constant of an exponential least squares fit to the duration histogram. Using suberyldicholine, the mean time constant of channel closing was 13 ms at 24 °C. This should be compared to 3 ms observed in tissue-cultured rat muscle with carbachol as an agonist⁵. Using fluctuation and voltage jump relaxation methods, Neher and Sakmann⁶ have estimated the mean channel open time at 18 °C in the frog to be 0.5 ms with carbachol and ~4 ms with suberyldicholine. Thus, it is clear that carbachol as an agonist is less efficient than suberyldicholine in keeping channels open. At the same temperature, both carbachol and suberyldicholine appear to keep channels open for relatively longer periods in homeothermic preparations than in poikilothermic preparations. When examined over a range of temperatures, the single-channel conductance and open time behave simply (Fig. 3). The variation of channel conductance with temperature is shown in Fig. 3a. Channel conductance

increases with increasing temperature with a Q_{10} of 1.3. The channel conductance variation with temperature is similar to the Q_{10} of 1.7 reported earlier from fluctuation analysis using acetylcholine⁴. Contrary to the results reported by Fischbach and Lass², we see no levelling off of the conductance at high temperatures, or any evidence of discontinuous change in conductance with temperature. The single-channel results are unambiguous in showing a high conductance at body temperature. The temperature dependence of the mean channel open time is shown in Fig. 3b. The enthalpy of activation, as calculated from the slope of the regression line, was 4.4 kcal mol⁻¹. If we combine data from fluctuation measurements⁴ and the present work, a curious pattern emerges whereby the activation enthalpy of the closing rate is inversely correlated with the open time. This may reflect entropic contributions to the activation energy for closing or perhaps a more complicated interaction of the rates for agonist binding and channel open-closed isomerisation.

Some records showed what appeared to be multiple gating of a single channel (Fig. 4). In these records the mean opening rate was low (duty cycle = 0.03) and hence there was a low probability of finding more than one channel open at a time. Nonetheless, we observed many events similar to those shown in Fig. 4 in which a channel apparently turns on and off several times before closing 'for good'. This burst-like behaviour is similar to that reported by Patlak *et al.*⁹ for desensitised channels and by Neher and Steinbach¹⁰ for endplate channels treated with local anaesthetics. As the opening rate and the agonist concentration were low, high probability agonist binding does not seem to be the cause of the multiple gating events. Perhaps suberyldicholine as a bivalent agonist, is able to block the same channels it activates¹¹, or the agonist dissociation rate is comparable to the channel opening rate so that several open-closed cycles may take place before agonist dissociation¹². Further work needs to be carried out to elucidate the mechanism of this repetitive behaviour.

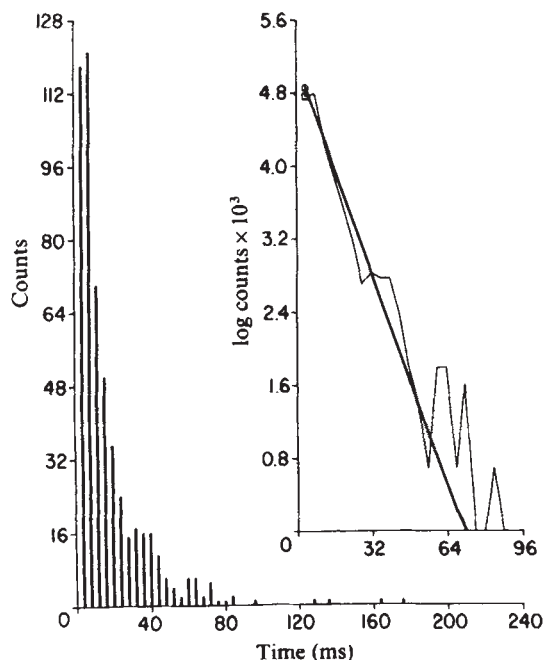


Fig. 2 Histogram of single-channel durations. Figure 1a is a typical segment of the data used to construct the histogram. Inset shows the same data plotted semilogarithmically and fitted with a single exponential (heavy line) by a least squares fit routine. Both amplitude and duration histograms were calculated from the raw data by an automatic pattern recognition program (F.S. and N. Barkakati, in preparation).

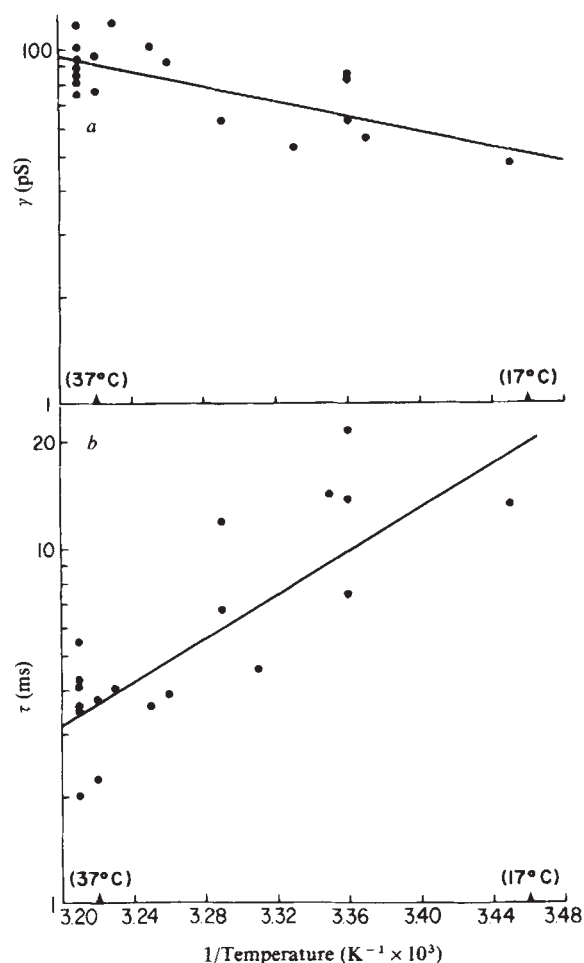


Fig. 3 Arrhenius plot of the single-channel conductance (a) and mean channel open time (b). Each point represents the results of measurements on an individual cell. a, Single-channel currents used to calculate conductance were obtained from amplitude histograms in accordance with a model which predicts the shape of the histogram and takes into account the fact that the wall thickness of the patch pipette is not negligible (channels which lie under the rim of the pipette do not contribute a full share of current to the observed record (F.S. and N. Barkakati, in preparation). The solid line is a least squares regression with a slope equivalent to an enthalpy of activation of 2 kcal mol⁻¹. b, Mean open times from individual cells were obtained from duration histograms. The line, which represents a least squares regression fit to the data, corresponds to an enthalpy of activation of 4.4 kcal mol⁻¹.

This work demonstrates that single-channel recording techniques can be easily extended to a wider variety of preparations than previously reported. More specifically, we find no anomalous temperature dependence of channel conductance. Results presented here were obtained on myotubes not treated with vinblastine, so that vinblastine treatment cannot account for the discrepancy between the results of Fischbach and Lass² and the results presented here. The origin of the discrepancy remains obscure, but we are reticent to ascribe the differences to

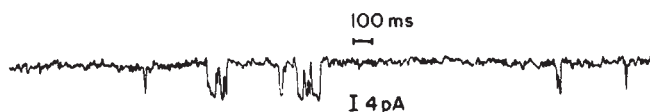


Fig. 4 Chart recording of membrane current illustrating the occurrence of apparent multiple gating of a single channel (see text for details). Temperature, 27 °C; bandwidth, 100 Hz.

the slight variations between the preparations, as these results agree reasonably well with the earlier results obtained with fluctuation analysis on vinblastine-treated cells, as well as with earlier data on myotubes using acetylcholine as an agonist¹³.

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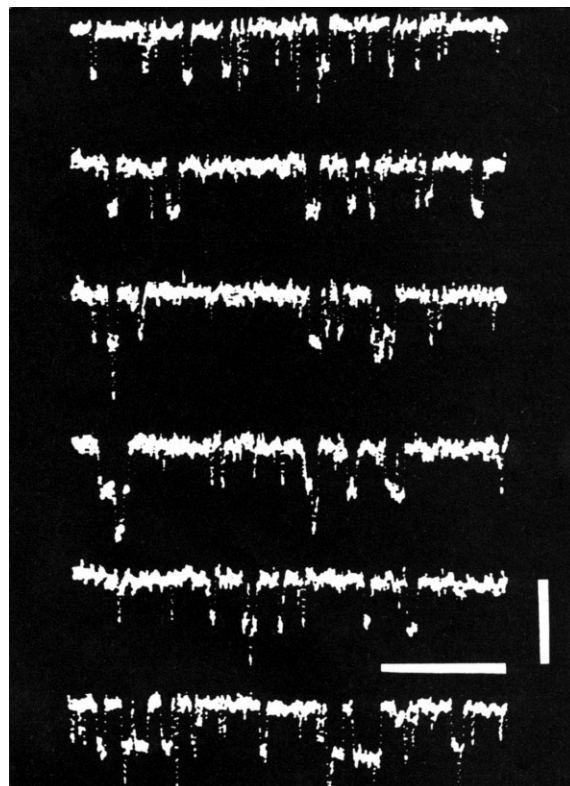


Fig. 1 Channel records made from cultured rat muscle at 22 °C. The muscle was bathed in Dulbecco's phosphate-buffered saline, with tetrodotoxin added to prevent spontaneous contraction. The currents were recorded from a thick-walled glass micropipette fabricated with a deFonbrune microforge. This micropipette was filled with saline and 1.0 μ M carbamylcholine. Its resistance was 3 M Ω and increased to 40 M Ω when pushed against the cell surface. The cell was hyperpolarised to -90 mV using two intracellular electrodes, one to pass current and the other to record the cell membrane potential. Scales: horizontal, 100 ms; vertical, 10 pA.

Single postsynaptic channel currents in tissue cultured muscle

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Some of the most compelling evidence for the existence of ionic channels in cell membranes comes from direct recording of quantised current jumps generated by the opening and closing of individual channels. Single-channel jumps have been extensively studied for lipid bilayer membranes doped with various channel-forming additives¹. Recently agonist-induced single-channel currents were detected in denervated frog muscle by use of extracellular electrodes, which can isolate the current from a small area of membrane². The current jumps provide a means for the direct test of many of the inferences about ionic channels which have come from electrical noise analysis^{3,4}. In this report we present measurements of single-channel currents induced by the agonist carbamylcholine in tissue-cultured mammalian muscle. These measurements confirm the earlier noise studies on tissue culture preparations^{5,6}. Recordings of single-channel currents induced by the agonist, suberyldicholine, in avian muscle are presented by Nelson and Sachs⁷.

The methods used for single-channel recording have been described by Neher *et al.*⁸. A small ($\sim 1 \mu\text{m}^2$) patch of cell surface is isolated with a specially fabricated extracellular glass microelectrode. A low-noise circuit amplifies the current through this patch of membrane. The signal is then recorded on a digital oscilloscope and can be stored on tape for later analysis. Dissociated embryonic rat muscle was grown according to the procedure of Nelson *et al.*⁹.

Figure 1 shows channels recorded from a cell held at a membrane potential of -90 mV. From such recordings, the single-channel conductance and the open-channel durations are measured directly. Figure 2a shows a histogram of the distribution of open-channel lifetimes. The number of channels remaining open for a time greater than T is plotted against T . The semilogarithmic plot of open-channel durations fits well to a single-exponential distribution, consistent with the notion that the closing of the channels is a process having a Poisson distribution. The mean channel open time at a temperature of 22 °C is 3.2 ms.

The mean single-channel current at -90 mV is 4.4 pA, indicating that the unit channel conductance is 49 pS (assuming a reversal potential of zero for the postsynaptic channel). The standard deviation obtained from the channel current amplitude distribution is 0.8 pA. The two main reasons for this spread are the intrinsic current noise of the wide bandwidth amplifier needed to record the short-lived events and the recording of undersized current jumps from channels situated beneath the annulus of the pipette.

As a check on the straightforward but tedious determination of individual channel amplitudes and durations, we also used simple automated procedures to obtain these parameters. Amplitude histograms were made directly from the data using a computer program. Figure 2b inset shows a current amplitude histogram using 4,096 points spaced 200 μ s apart. The direct histogram shows three peaks, a large gaussian distribution of baseline noise centred at zero, a second peak about the single channel amplitude, and a third smaller peak at twice the single-jump amplitude. The channel current can be estimated from the displacement of the secondary peaks without the necessity of analysing individual events. The weighting of events is different here than for the direct measurement, as a long-lived event will contribute more points to the histogram than a shorter event. Using the amplitude histograms, we obtained a single-channel current-voltage curve (Fig. 2b). The plot of current peaks versus applied membrane potential is linear over the voltage range measured. From the slope of the line, we obtain a unit conductance of 48 ± 0.4 pS.

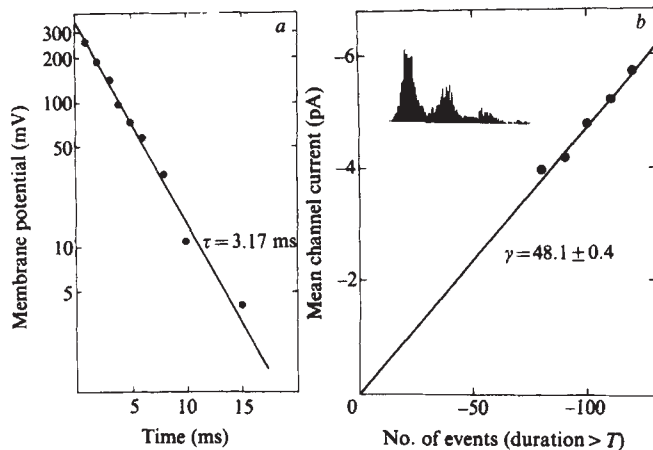


Fig. 2 *a*, A semilogarithmic plot of the number of events with duration greater than time T plotted against T shows the exponential probability of channel open time. The line is a least squares fit to the data. *b*, Single-channel current-voltage curve. Abscissa gives holding potential with increasing hyperpolarisation to the right. Ordinate shows inward current as determined from the second peak of current histogram. Inset, a typical current histogram, taken at a holding potential of -100 mV. The histogram is determined from a record of 4,096 points taken at 200- μ s intervals; the horizontal distance between the first two peaks is 4.8 pA and the vertical displacement is proportional to the number of points with a given amplitude.

To analyse channel lifetimes automatically, a Fast Fourier Transform program was used to determine the power spectra of digitised records similar to those shown in Fig. 1, but usually with a higher density of channel events. The power spectra are lorentzian, as expected from the underlying Poisson random process. The mean channel durations determined from the cutoff frequencies of the noise spectra were in agreement with the values determined from the pulse-duration histograms. Thus, spectral analysis methods can be used as a convenient method to analyse stationary records of single-channel jumps.

Single-channel measurements seem to be feasible for a variety of tissue-cultured excitable cells, and the patch-electrode method allows one to observe the ion channel activity in an area of a heterogeneous membrane of less than $5 \mu\text{m}^2$. Preliminary measurements carried out in collaboration with C. N. Christian indicate that the distribution of channel density determined with an extracellular patch electrode coincides with that obtained by observing the same cells in a fluorescence microscope using rhodamine labelled α -bungarotoxin¹⁰.

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Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9

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The transposable genetic element Tn9 consists of two direct repeats of the insertion sequence IS1 flanking a region of 1,102 base pairs which determines chloramphenicol resistance^{1,2}. Transposition of Tn9 leads to the duplication of a 9-base pair sequence which preexists at the site of insertion. One copy of this sequence is found at each end of the inserted element³. The chloramphenicol resistance determined by Tn9, and by various other R plasmids, is due to the synthesis of the enzyme chloramphenicol acetyl transferase (CAT)^{4,5}. This enzyme catalyses the formation of acetylated derivatives of chloramphenicol which are inactive as inhibitors of protein synthesis⁵. By using the chain termination technique of DNA sequencing, we have now determined the nucleotide sequence of the 1,102 base pair region between the directly repeated IS1 sequences in the bacterial transposon Tn9 (encoding chloramphenicol resistance). The amino acid sequence of CAT predicted from the nucleotide sequence is identical to that determined by Shaw and coworkers⁶. An analysis of the sequence suggests that the internal 1,102 base pair region is not directly involved in transposition.

Restriction endonuclease analysis of bacteriophage P1Cm DNA demonstrated that Tn9 contained unique *Pst*I cleavage sites within the directly repeated IS1 sequences but no *Pst*I sites within the chloramphenicol resistance determinant⁷. Cleavage of bacteriophage P1Cm with *Pst*I generated a fragment of 1,870 base pairs. This fragment contained the 1,102-base pair region encoding the structural gene for CAT flanked by 588 base pairs from the right end of IS1 and 180 base pairs from the left end of IS1. The 1,870-base pair fragment was cloned in the *Pst*I site of plasmid pBR322 generating pDV801 which served as the source of DNA in these studies (Fig. 1). A restriction map of the 1,102-base pair region is presented in Fig. 2.

To determine the nucleotide sequence of the 1,102-base pair region of Tn9 bounded by the IS1 sequences we used the rapid dideoxy chain termination technique developed by Sanger *et al.*⁸. Two methods were used to prepare the required single-stranded templates. One of these is exonuclease III digestion of double-stranded DNA⁹. This enzyme removes nucleotides from the 3' ends of double-stranded DNA. Therefore, exonuclease III digestion of a linear double-stranded molecule will generate a single-stranded region which may be used as a template in the primed synthesis reaction (Fig. 3). A typical gel using templates prepared in this manner is shown in Fig. 4. The other method of obtaining single-stranded templates involves molecular cloning in the single-stranded filamentous bacteriophage M13 (ref. 10). Infection of susceptible strains (sex-factor carrying strains of *Escherichia coli* K12) with M13 results in the establishment of a carrier state in which the double-stranded replicative form (RF) of M13 is maintained at about 300 copies per cell, while mature phage, containing one of the complementary strands (+) strand), is extruded into the medium^{11,12}. It has recently been demonstrated that foreign DNA can be inserted into and stably maintained in the M13 RF (refs 10, 12–14). Since the limit to the size of the DNA packaged by filamentous phage appears to be very large, the inserted DNA can be packaged as single-stranded DNA covalently linked to the phage DNA. This single-stranded DNA can then be isolated and used as a template for the dideoxy sequencing method. By cloning the DNA in both

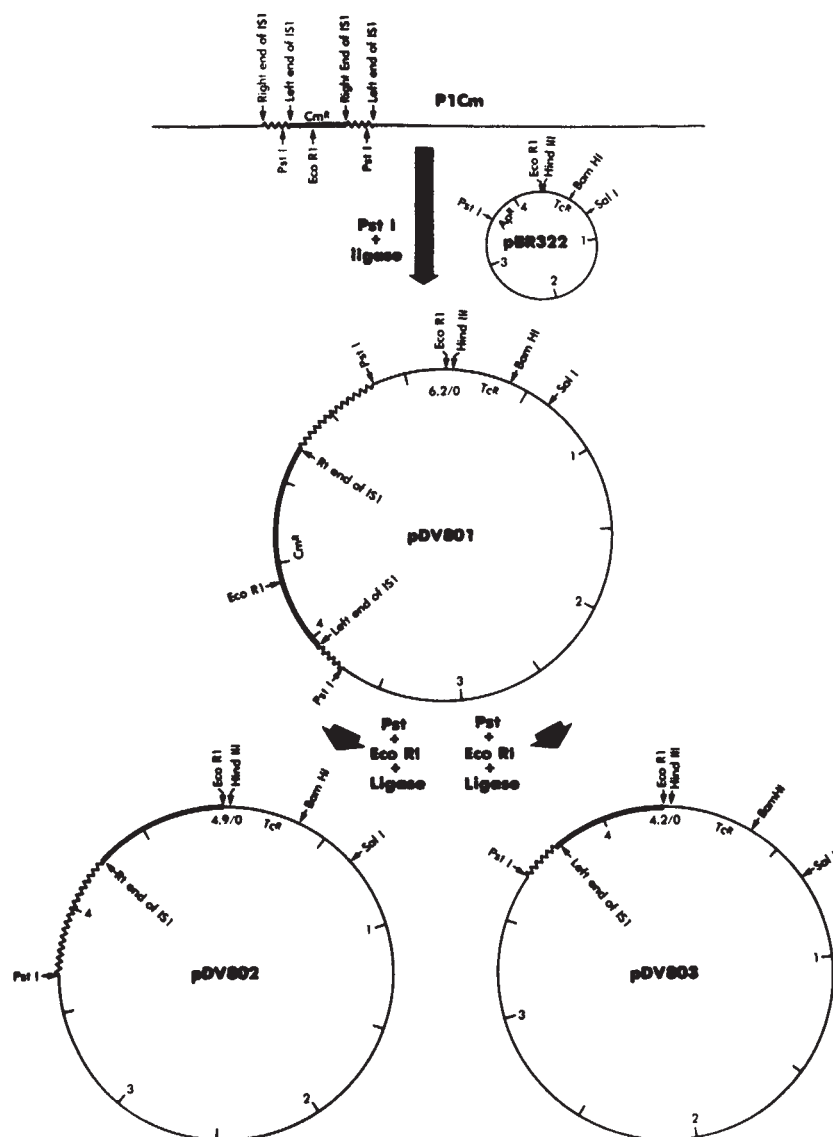


Fig. 1 Schematic representation of plasmid constructions. The derivation of pDV801 is shown and was previously described³⁶. pDV802 and pDV803 were constructed by digestion of pDV801 with *Pst*I and *Eco*RI, treatment with T4 polynucleotide ligase, and transformation of *E. coli* C600 using published procedures³⁷. The plasmids carried by several tetracycline-resistant, chloramphenicol sensitive strains were isolated and the fragments of Tn9 they contained determined by digestion with *Eco*RI plus *Pst*I. pDV802 carries a 1,254 base-pair fragment containing the right end of IS1 while pDV803 carries a 616 base-pair fragment containing the left end of IS1. Wavy lines indicate IS1 sequences. The numbers within the circles refer to distances in kilobases.

orientations, either of the complementary strands can be isolated (Fig. 5a). An example of a sequencing gel using M13 templates is shown in Fig. 5b.

In initial sequencing experiments, the exonuclease III method was used to prepare templates. During this work the M13 method became available and was used in completing the sequence. For both methods, DNA fragments generated by restriction endonuclease digestion of the 1,870-base pair fragment of Tn9 were used as primers (Fig. 2).

Using these techniques we have determined the nucleotide sequence of the 1,102-base pair region of Tn9 bounded by the directly repeated IS1 sequences (Fig. 6). In most regions both of the complementary strands were sequenced several times (Fig. 2). Amino acid sequence data of the CAT gene allowed further confirmation of the sequence as shown in Fig. 6 (ref. 6).

We have also sequenced approximately 200 base pairs within IS1 including 40 base pairs at the right end and 70 at the left end. The sequence of these regions is identical to that determined by Ohtsubo and Ohtsubo¹⁵ and Johnsrud¹⁶.

Three unique restriction endonuclease cleavage sites are located within the CAT coding sequence. These are an *Eco*RI site (nucleotide 437), a *Bal*I site (nucleotide 704), and a *Pvu*II site (nucleotide 337) (Fig. 6). These sites are potentially useful for DNA cloning since insertion of DNA into these sites should lead to the loss of chloramphenicol resistance, thereby simplifying the screening of recombinants. Two cloning vehicles which carry the CAT gene, pBR325 and pACYC184, have been constructed and used as *Eco*RI vectors^{17,18}. pBR325, however,

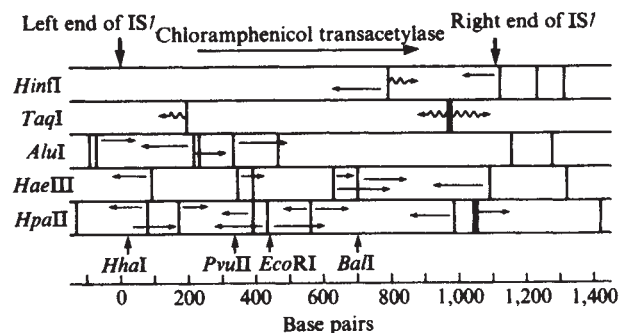


Fig. 2 Map of restriction endonuclease sites and sequencing strategy of the 1,102 base-pair region of Tn9 contained between the IS1 sequences. Positions of the endonuclease cleavage sites were determined using the technique described by Smith and Birnstein³⁸ and by analysis of multiple digestion products as previously described³⁷. Restriction endonucleases were obtained from New England Biolabs. Cleavage with restriction endonucleases *Hae*III, *Hpa*II, *Alu*I, *Taq*I and *Hha*I was carried out in H buffer which contains 6.6 mM Tris-HCl, pH 7.5, 6.6 mM MgCl₂, 6.6 mM NaCl, 5 mM dithiothreitol. Cleavage with restriction endonucleases *Pst*I, *Eco*RI and *Hin*I was in H buffer plus 50 mM NaCl. All reactions were at 37 °C with the exception of *Taq*I which was at 60 °C. The position of the *Bal*I site is inferred from the nucleotide sequence. Vertical lines indicate cleavage sites for the endonucleases shown at the left. Arrows show the direction and extent of sequencing runs. Wavy arrows indicate sequencing runs performed using the Maxam and Gilbert technique⁴⁰. The left and right ends of IS1 are indicated.

has an additional *PvuII* site as well as an additional *BalI* site. These sites would have to be removed to make appropriate cloning vehicles for use with these restriction endonucleases.

Since the nucleotide sequences surrounding these sites are now known, they are also potentially useful for the formation of fused proteins. These fused proteins would then be under the control of the CAT promoter. It has recently been demonstrated that the CAT gene carried by pBR325 can be expressed in *Saccharomyces cerevisiae* (J. Cohen and R. Needleman, personal communication). Therefore, it would be feasible to test the expression of these fused proteins in yeast as well as in *E. coli*.

Analysis of the nucleotide sequence reveals an uninterrupted coding sequence for 219 amino acids beginning with the AUG initiator codon at nucleotide 224 and terminating with the nonsense codon UAA at nucleotide 881. This amino acid sequence is identical to that determined by Shaw *et al.* for the Type I CAT isolated from strains carrying plasmid JF66b (see the accompanying paper)⁶.

Shine and Dalgarno¹⁹ suggested that ribosome recognition of mRNA involves complementary base-pairing between the 3' end of the 16S rRNA and the 5' end of mRNA. Such complementarity has been confirmed by sequence analysis of a number of different genes²⁰⁻²². Two such sequences are found preceding the initiation codon for the CAT gene. These are AGGAG

(nucleotides 205-209) and TAAGGA (nucleotides 211-216). Based on its spacing from the AUG initiator codon, the sequence TAAGGA is most likely involved in ribosome recognition. However, it is possible that the AGGAG sequence might also have some role.

The sequence data in Fig. 6 show that the amino terminal codon of the CAT gene is located 224 nucleotides from the left end of IS1. Assuming that the CAT promoter is not in IS1, these 224 nucleotides encode the regulatory functions of the CAT gene. The level of expression of the CAT gene is regulated by the cyclic AMP receptor protein (CAP) and cyclic AMP^{23,24}. The promoter regions of the lactose and galactose operons which are also regulated by CAP plus cyclic AMP contain a sequence, referred to as the CAP site, which precedes the start point of transcription (for reviews see refs 25, 26). This region presumably interacts with the CAP-cyclic AMP complex stimulating the initiation of transcription. Biochemical and genetic data have demonstrated that the CAP site in the *lac* promoter contains the sequence GTGAG---CACTC which is involved in binding the CAP-cyclic AMP complex²⁵. Similar sequences are found in the promoter regions of the galactose and *araBAD* operons^{27,28}. Inspection of the DNA sequence between the left end of IS1 and the start of the CAT coding sequence reveals a similar sequence GTGA---TCAC (Fig. 6, nucleotides 29-43). By analogy with *lac*, *gal* and *araBAD* this region may be

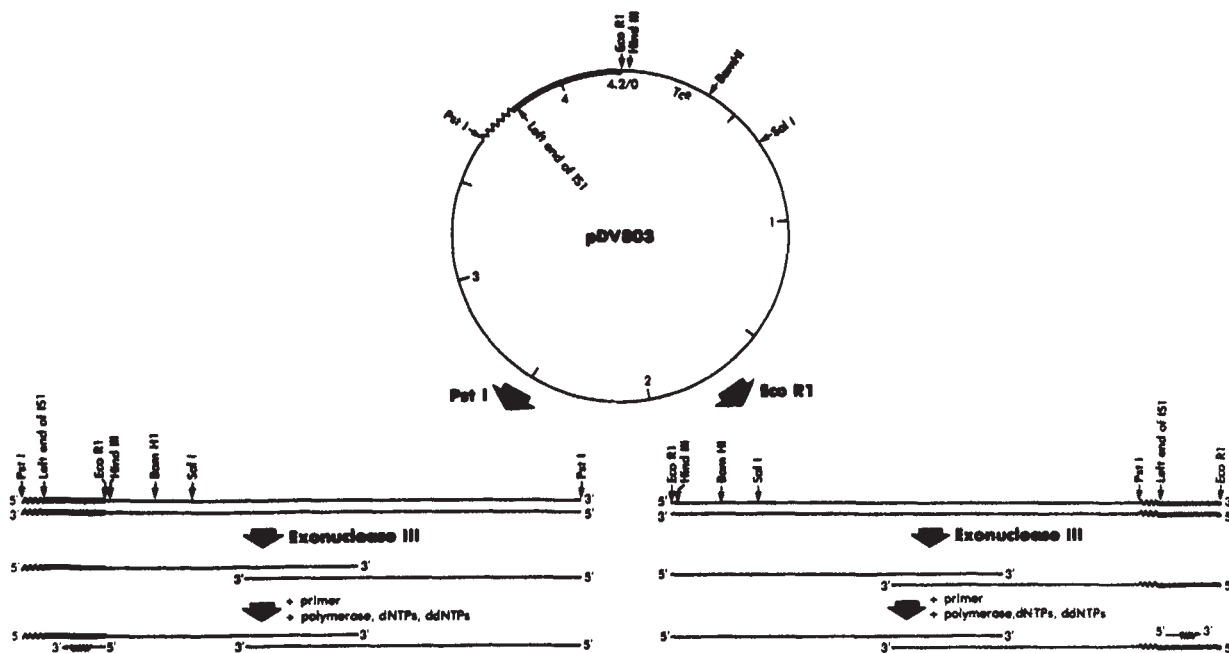
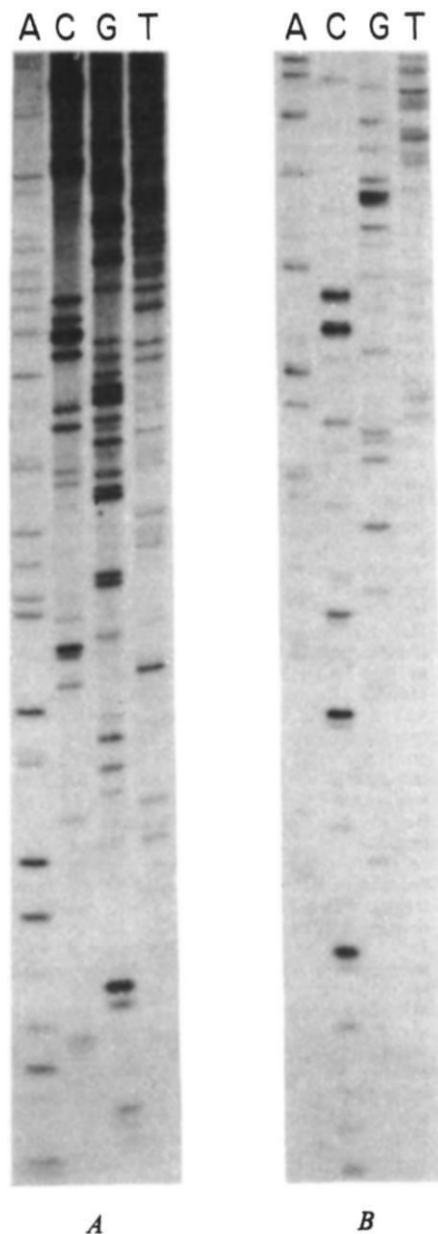


Fig. 3 Exonuclease III preparation of templates for the primed synthesis reactions. The most useful templates prepared by exonuclease III treatment were those containing the DNA to be sequenced at the end of the molecule. Therefore, plasmids to be used for preparing exonuclease III templates should contain unique restriction cleavage sites adjacent to the region to be sequenced. To prepare such plasmids we subcloned the fragments of Tn9 generated by digestion of pDV801 with *EcoRI* plus *PstI*. The two plasmids derived from this cloning, pDV802 and pDV803, have unique *PstI* and *EcoRI* sites (Fig. 1). The use of one of these plasmids, pDV803, to prepare single-stranded templates is outlined above. Cleavage with *PstI* followed by exonuclease III digestion exposes one strand of Tn9 while cleavage with *EcoRI* followed by exonuclease III treatment exposes the opposite strand. By utilising both of these templates the sequence of the complementary strands could be determined. DNA prepared in this manner was suitable as a template using restriction endonuclease generated fragments less than 100 base pairs as primers. Larger fragments were not as useful since they must be cleaved with restriction endonucleases from the extended chains before gel electrophoresis. This cleavage step was necessary to obtain chains small enough (less than about 200 base pairs) to be resolved on the sequencing gel. However, cleavage of primers when using exonuclease III prepared templates led to artefactual bands in all lanes of the gel, probably due to residual double-stranded regions in the DNA template which could either be nick-translated or whose 3'OH ends could act as self-primers. In either case, restriction endonuclease digestion would release radioactively labelled chains. If these chains were of a size which would migrate within the resolving limits of the sequencing gel, they would appear as bands in all channels. Smith⁹ found that this problem could be eliminated by more extensive treatment with exonuclease III. However, in our hands, longer exonuclease digestion led to degradation of the DNA and loss of template activity. Although such degradation of the single-stranded DNA might be due to the endonuclease III activity known to be associated with exonuclease III, this is unlikely since the endonuclease II activity has been shown to be specific for damaged DNA³⁹. Another possibility is that some other nuclease was present in the preparation of exonuclease III used in these experiments. Exonuclease III can also be used for the preparation of primers (R. J. Roberts, personal communication). Double-stranded restriction endonuclease generated fragments were exhaustively digested with exonuclease III, and the products of this reaction used as primers with the M13 templates described in Fig. 5. Analysis of sequences determined by this technique demonstrated that the double-stranded fragment had been reduced to single-stranded primer of approximately 15 bases. If the double-stranded fragments treated with exonuclease III for use as primers are small (less than the resolving limits of the sequencing gel), sequences adjacent to the fragment can also be determined.

Fig. 4 Autoradiogram of a sequencing gel using exonuclease III treated templates and primers. Plasmid DNA was isolated as previously described and purified by isopycnic centrifugation in CsCl-ethidium bromide gradients³⁷. The DNA was further purified by velocity sedimentation in a 5–20% neutral sucrose gradient containing 1 M NaCl, 0.1 M Tris-HCl, pH 7.0. 100–200 µg of DNA was layered on a 5 ml gradient and centrifuged for 3.5 h at 40,000 r.p.m. in a SW50.1 rotor. Peak fractions containing the DNA were pooled and the DNA precipitated by the addition of 2.5 volumes of 95% ethanol, pelleted by centrifugation (40,000 r.p.m., 20 min), resuspended in distilled water and stored frozen at –20°C. For exonuclease III treatment, 10 µg of DNA was digested with either *Pst*I or *Eco*RI (H buffer + 50 mM NaCl) in 25 µl followed by incubation for 10 min at 65°C to inactivate the restriction endonucleases. The DNA was then dialysed for 2 h against 200 ml of exonuclease III buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM dithiothreitol (DTT)). The volume was adjusted to 50 µl and 10 units of exonuclease III (New England Biolabs, 1 unit per µg DNA) was added. After incubation for 45 min at 37°C, an additional 10 units of exonuclease III was added and incubation was continued for 30 min at 37°C. Exonuclease III was inactivated by a 15 min incubation at 65°C and the DNA was stored at –20°C. For use as a template, 0.1 pmol of exonuclease III-treated DNA was mixed with 0.5 pmol of restriction fragment primer prepared as described below in a final volume of 10 µl containing H buffer. This mixture was then sealed in a glass capillary, boiled for 3 min and hybridised at 68°C for 30 min. 2 µl of this mixture was used in each reaction as described below. Extension with *Poll* (Klenow fragment, New England Biolabs) in the presence of deoxynucleotide triphosphates and dideoxynucleotide triphosphates was performed essentially as described by Sanger *et al.*⁸. Reactions were carried out in 1.5 × H buffer containing 0.5 µM [α -³²P]dATP (400 Ci mmol⁻¹, Amersham Radiochemicals) and nucleotides at the following concentrations:

ddA: 35 µM dCTP; 35 µM dGTP; 35 µM dTTP; 50 µM ddATP
 araC: 3.5 µM dCTP; 35 µM dGTP; 35 µM dTTP; 5000 µM araCTP
 ddC: 3.5 µM dCTP; 35 µM dGTP; 35 µM dTTP; 200 µM ddCTP
 ddG: 35 µM dCTP; 3.5 µM dGTP; 35 µM dTTP; 200 µM ddGTP
 ddT: 35 µM cCTP; 35 µM dGTP; 3.5 µM dTTP; 200 µM ddTTP

All reactions were performed in glass capillaries in a final volume of 5 µl containing 0.2 units of DNA polymerase (Klenow fragment). After incubation for 15 min at room temperature, 1 µl of 0.5 mM dATP was added to each reaction and incubation was continued for 5 min at room temperature. If restriction endonuclease cleavage was necessary, 0.2 µl of restriction endonuclease was added to each reaction and incubated at 37°C for 5 min. 10 µl of dye mix (90% deionised formamide, 0.25% bromophenol blue, 0.25% xylene xanol) was added and the reactions were boiled for 3 min prior to gel electrophoresis. If restriction endonuclease cleavage was not necessary, the dye mix was added immediately following the dATP chase step. **Panel A**, pDV803 was prepared as a template by cleavage with *Pst*I followed by treatment with exonuclease III as described above. The primer was a 14-base pair DNA fragment isolated from a multiple digestion (*Hae*III + *Hpa*II + *Alu*I) of the 616-base pair fragment of Tn9 (isolated from pDV803). The products of this reaction were fractionated on a 40 cm × 20 cm × 1.5 mm 12% polyacrylamide gel containing 25% glycerol in TB buffer. After electrophoresis, the gel was stained with ethidium bromide; individual bands were cut out and the DNA eluted from the acrylamide by soaking in 1.2 ml of buffer (0.3 M NaOAc, 0.1 M Tris-HCl, pH 8.0, 2.5 mM EDTA) for 16 h at 37°C. The DNA fragments were then precipitated by addition of 2.5 volumes of ethanol, centrifuged in a SW50.1 for 30 min at 30,000 r.p.m., resuspended in distilled water and stored frozen at –20°C. Primer-template hybridisation and chain elongation were as described above. There was no cleavage step with restriction endonuclease after extension with DNA polymerase (Klenow fragment). Sequence begins at nucleotide 73 and proceeds to the left (complement to the strand shown in Fig. 6). **Panel B**, 1.0 pmol of a 420-base pair DNA fragment generated by digestion with endonuclease *Hae*III was incubated in a volume of 15 µl containing 4 units of exonuclease III in 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM MgCl₂ for 1 h at 37°C. The mixture was then sealed in a capillary and boiled for 3 min. 5 µl of this mixture (0.33 pmol) was annealed with 0.1 pmol of M13pCmA DNA and used as a substrate for the primed synthesis reaction as described above. Electrophoresis in both panels was for 2 h at 35 W constant power on an 8% polyacrylamide–7 M urea gel (40 cm × 20 cm × 0.45 mm) as described by Sanger and Coulson⁴¹. The sequence begins at nucleotide 1,070 and proceeds to the left (complement to the strand shown in Fig. 6).



involved in the interaction with CAP–cyclic AMP complex. The CAP sites of the *lac*, *gal* and *araBAD* promoters are bounded by AT-rich sequences. Similarly, the putative CAP site in the CAT promoter is bounded by direct repeats of the AT-rich sequence GAATAAATA (Fig. 6, position 17–25 and position 50–58). Whether these sequences are also involved in the CAP–cyclic AMP interaction is not known.

Other features common to promoters are sites of the molecular interaction with RNA polymerase. Two common sites have been defined from mutational and sequence analysis of a number of different promoters. One of these is the heptanucleotide TATPuATPu (Pribnow box) which is centred approximately 10 nucleotides before the start point of transcription²⁹. The other is the so-called –35 region which is centred approximately 32 nucleotides before the start point of transcription³⁰. Although several such sequences are present in the CAT promoter, assignment of a specific role to these sequences awaits determination of the 5' termini of the CAT mRNA(s).

The *ochre* codon at nucleotide 881–883, which is presumably the translational stop for the CAT gene, is immediately followed

by the sequence TTTTTTTAA and preceded by a GC-rich region (13 of 14 base pairs). This region may represent the CAT mRNA termination site. This assumption is based by comparison with other genes in which similar sequences have been observed at the transcriptional termination site (for review, see ref. 31). However, the inverted repeat sequences found in these other instances (characteristic of ρ -dependent termination) which can potentially form stem-loop structures, are not found in the CAT DNA sequence.

Of the 1,102 base-pair region of Tn9 bounded by the IS1 sequences, at least nucleotides 28–883 are involved in the expression of CAT. An analysis of the sequence distal to the CAT translational stop signal reveals an uninterrupted coding sequence for 75 amino acids beginning with an AUG initiator codon at nucleotide 965 and extending 81 nucleotides into IS1. No other protein larger than 40 amino acids can be predicted from translation of all three reading frames of either strand of the 1,102 base-pair region.

Arber *et al.* have shown that Tn9 carries fusidic acid resistance as well as chloramphenicol resistance³². Based on the sequencing data, fusidic acid resistance must be due to either

Fig. 5 a, Schematic representation of Tn9 fragments cloned in M13pbla6. A derivative of M13 Tn-15 was used as a cloning vehicle¹³. This phage, M13pbla6, contains the β -lactamase gene from Tn3 inserted in the intercistronic region between genes II and IV of M13 (D. Ray and S. Hines, personal communication). Strains which harbour M13pbla6 are therefore ampicillin resistant. The single *Pst*I site in M13pbla6 was used to clone the 1,870 base-pair fragment of Tn9 generated by digestion with *Pst*I. Since the *Pst*I site of M13 is within the β -lactamase gene, insertion of the 1,870 base-pair fragment at this site resulted in phages which conferred the phenotype chloramphenicol resistance-ampicillin sensitivity on infected cells. RF DNA was isolated from infected cells with this phenotype as described below and analysed by restriction endonuclease digestion. Of 28 independent isolates screened, 26 carried the inserted fragment in one orientation (M13pCmA) and only two were in the other orientation (M13pCmB). Barnes¹⁴ also noted an orientation effect in cloning the *His* operon DNA into M13. In that instance a slower growth rate and a lower yield of phage was observed when DNA was inserted in one particular orientation. Phage DNA was isolated from M13pCmA and M13pCmB and used as templates with primers prepared from restriction endonuclease generated fragments of pDV801, pDV802, and pDV803. Since the template is completely single-stranded, no template related problems were encountered when the primers were recleaved from the extended chains with restriction endonucleases. However, successful cleavage with restriction endonucleases was dependent upon the particular lot of endonuclease used. M13pbla6 was purified from infected cells as described by Messing¹⁰. 0.5 μ g of this DNA was cleaved with *Pst*I and mixed with 0.35 μ g of the 1,870 base-pair DNA fragment of Tn9 (purified from pDV801). Ligation with T4 polynucleotide ligase was performed in a final volume of 50 μ l of H buffer (see legend to Fig. 2) plus 0.4 mM ATP for 16 h at 5 °C. 0.5 μ l of this mixture was used to transfect 0.1 ml of Ca²⁺ treated *E. coli* K802F' lac (ref. 10). The transfected cells were mixed with 0.2 ml of uninfected cells (4×10^7 cells) and overlayed on YT plates. Infected cells from individual plaques were tested for chloramphenicol resistance. Small cultures were grown from chloramphenicol-resistant infected cells and RF DNA was isolated as previously described for plasmid screens³⁷. The RF DNA was digested with *Pst*I to determine whether the Tn9 fragment was present, and with *Eco*RI plus *Hind*II to determine the orientation of the inserted fragment. The two orientations (M13pCmA and M13pCmB) are shown schematically. The numbers indicate the size of the phage DNA in kilobases. To prepare M13pCmA and M13pCmB single-stranded DNA, the supernatant from a litre culture of infected cells grown in YT medium was adjusted to 0.5 M NaCl and the phage precipitated by the addition of polyethylene glycol to a final concentration of 4%. Precipitated phage were collected by centrifugation and resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂. After a low speed spin (10,000 r.p.m. for 10 min) to remove cellular debris, the phage was lysed by heating in 0.5% SDS, 50 mM EDTA for 10 min at 65 °C. The phage DNA was extracted with phenol followed by extraction with phenol-chloroform and precipitated with 2.5 volumes of 95% ethanol. The DNA was further purified by centrifugation for 3 h in a 5–20% neutral sucrose gradient as described in the legend to Fig. 4. For use as a template, 0.1 pmol of DNA was used and the reactions carried out as described in the Fig. 4 legend. The arrows indicate the direction of chain extension after annealing with primers. **Fig. 5b**, Autoradiogram of a sequencing gel utilising M13pCmA or M13pCmB templates. pDV802 or pDV803 (6 pmol) were cleaved with *Pst*I plus *Eco*RI in H buffer containing 50 mM NaCl. The DNA was precipitated with 2.5 vol ethanol and resuspended in 30 μ l of distilled water. 2.5 μ l (0.5 pmol) of this DNA was mixed with 0.1 pmol of template (M13pCmA or M13pCmB) prepared as described above. After boiling for 3 min in a sealed capillary, annealing and chain elongation were carried out as described in the Fig. 4 legend. After incubation with *Pol*I and a 5 min chase with 1 μ l of 0.5 mM dATP, 0.2 μ l (10 units) of *Eco*RI (panels A and B) was added and the incubation continued at 37 °C for 5 min before addition of the dye mix. Electrophoresis on a 8% polyacrylamide–7 M urea gel was as described in the legend to Fig. 4. **Panel A**, M13pCmA template, pDV802 cleaved with endonucleases *Pst*I plus *Eco*RI as primer. Sequence begins at nucleotide 412 and proceeds to the left (complement to strand shown, Fig. 6). **Panel B**, M13pCmB template, pDV803 cleaved with endonuclease *Pst*I plus *Eco*RI as primer. Sequence begins at nucleotide 470 and proceeds to the right (strand shown in Fig. 6). **Panel C**, M13pCmB template, 105-base pair *Alu*I generated DNA fragment primer. After extension and chase, 0.2 μ l *Pvu*II was added to release the extended chains. Reaction conditions and primer purification were as described in the legend to Fig. 4. Sequence begins at nucleotide 373 and proceeds to the right (strand shown in Fig. 6).

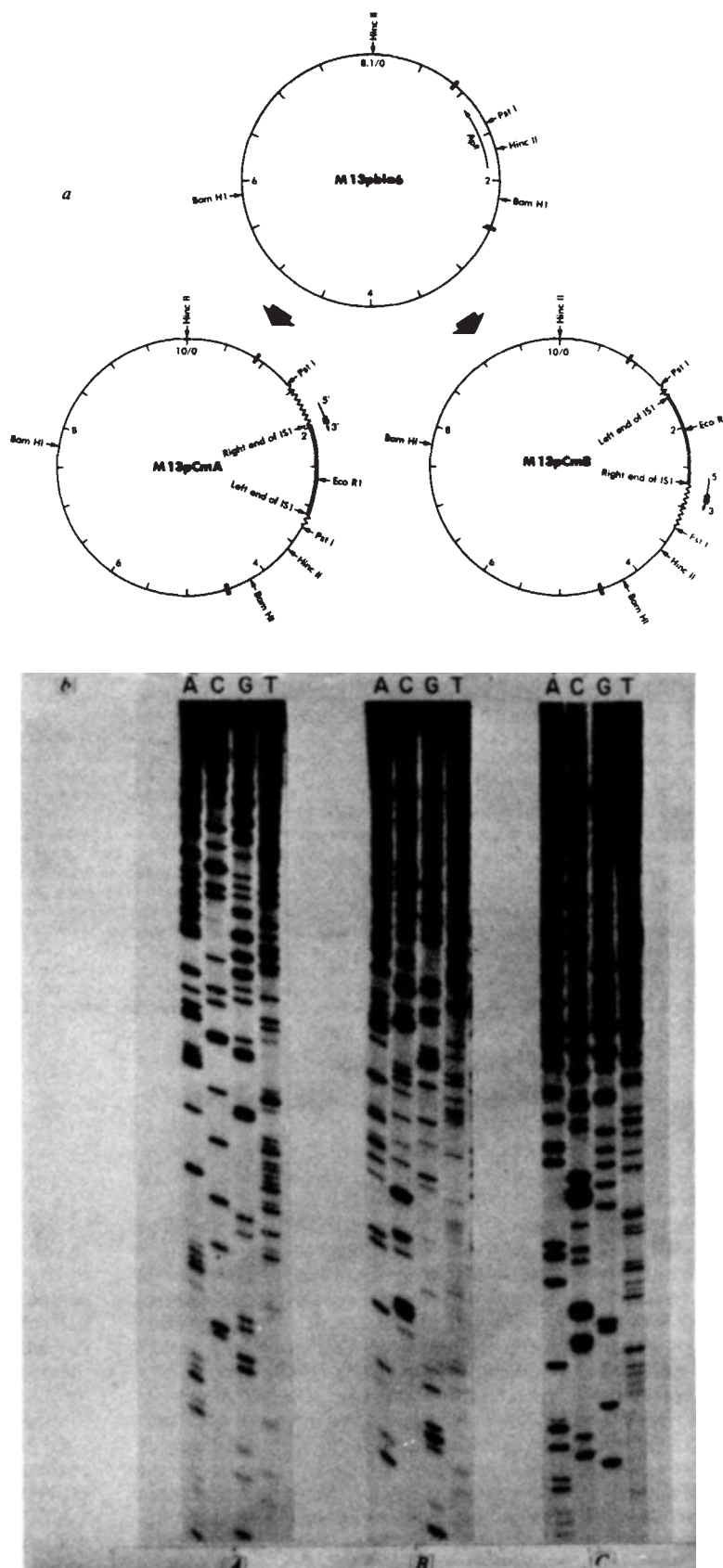


Fig. 6 Nucleotide sequence of the 1,102 base-pair region of Tn9 between the direct repeats of IS1. The amino acid sequence of chloramphenicol acetyl transferase predicted from the nucleotide sequence is indicated above the respective codons. Numbering of nucleotides beginning at the left end of IS1 (designation of Ohtsubo and Ohtsubo, ref. 15) is as shown. The junctions with IS1 are indicated by a colon (:). 20 nucleotides from both the left and right ends of the IS1 sequences present in Tn9 are also shown.

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Primary structure of a chloramphenicol acetyltransferase specified by R plasmids

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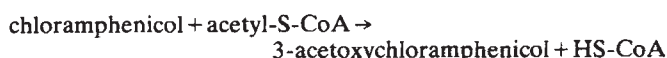
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Naturally occurring isolates of chloramphenicol-resistant bacteria commonly synthesise chloramphenicol acetyltransferase (EC 2.3.28; CAT) in amounts which are sufficient to account for the resistance phenotype and often harbour plasmids which carry the structural gene for CAT^{1,2}. The finding of CAT in such diverse prokaryotes as *Proteus mirabilis*, *Agrobacterium tumefaciens*, *Streptomyces* sp., and a soil *Flavobacterium* has led to speculation concerning the origin and evolution of the more commonly observed CAT variants specified by plasmids in clinically important bacteria². To provide a more solid basis for studying the evolution and spread of CAT within prokaryotes we chose to determine the complete amino acid sequence of a type I variant of CAT, the variant known to be associated with most F-like plasmids conferring chloramphenicol resistance. The sequence has been determined by combining the results obtained from manual and automated sequential degradation with those obtained by mass spectrometry of peptides generated by enzymatic digestion. The directly determined primary structure is identical with that predicted by the DNA sequence analysis³ of the chloramphenicol resistance transposon Tn⁹ known to specify a type I variant of chloramphenicol acetyltransferase.

CAT catalyses the acetylation of chloramphenicol to yield as the initial product 3-acetoxychloramphenicol which is devoid of significant antibiotic activity⁴:



The plasmid-borne chloramphenicol resistance conferred by CAT in *Escherichia coli* and other related Gram-negative bacteria is a constitutive property of such strains whereas staphylococci synthesise CAT only after exposure to the antibiotic or closely related compounds including a gratuitous inducer which neither serves as a substrate for CAT nor is an antibiotic⁵. Three reasonably well defined and divergent types of CAT have been shown to be mediated by plasmids in Gram-negative bacteria whereas staphylococcal plasmids appear to specify at least four closely related variants^{6,7}.

All CAT variants studied to date appear to exist in solution as tetramers of four identical subunits⁸. The CAT monomers of all variants possess molecular weights in the range 22,000–24,000 by SDS-polyacrylamide gel electrophoresis. In the light of the calculated molecular weight for the complete 219 amino acid primary structure of the enzyme described below, it seems likely that the expected monomeric molecular weights of most if not all CAT variants will need to be revised upwards.

The yield of pure protein was maximised without recourse to recombinant technology by taking advantage of several intrinsic advantages of the system. High level chloramphenicol resistance mutants can be isolated by selection for other plasmid-linked drug resistance markers, yielding high copy number plasmids⁹. Furthermore, the expression of CAT is known to be subject to cyclic AMP-mediated catabolite repression and, hence, growth of bacterial cells on glycerol rather than glucose can lead to up to

fivefold increases in yield of CAT¹⁰. Finally, the development of a reproducible high capacity affinity chromatographic method for the purification of CAT has given almost quantitative recovery of electrophoretically homogeneous material in a single purification step¹¹. One gramme of pure CAT specified by plasmid JR66b was used for most of the studies described. Supplementary information has been provided by structural studies on smaller amounts of a closely related type I variant specified by plasmid R429 (ref. 12).

The initial approach to determining the complete covalent structure of CAT took account of several preliminary observations. The protein was known to contain not less than seven methionines, one of which was N-terminal and unblocked. Although tryptic digests of the reduced and carboxymethylated protein yielded useful peptide maps, there were regions of structure not likely to be represented in soluble peptides. By contrast and as expected, digests of CAT with chymotrypsin and elastase went smoothly. The overall plan was therefore as follows: (1) determine the N-terminal amino acid sequence by automated Edman degradation; (2) prepare and sequence fragments isolated after cleavage at methionines with cyanogen bromide, using both automated Edman degradation and enzymic sub-digestion followed by manual 'dansyl' Edman methods; and (3) explore the use of mass spectrometry (MS) to determine the amino acid sequence of fragments generated by chymotrypsin, elastase and subtilisin. A particular advantage of the use of mass spectrometry was not only the relative speed with which the sequence of many small (three to nine residue peptides can be determined but also the attraction of 'mixture analysis' to get sequence information from column or paper fractions containing several peptides¹³. In addition to these three main approaches we also took advantage of the results obtained by dansyl Edman and automated sequence analysis of certain tryptic peptides and results of manual sequencing of chymotryptic or elastase peptides to extend the information from the mass spectrometry results. The techniques used have been described in detail elsewhere^{13–18}.

The complete amino acid sequence of CAT is shown in Fig. 1. The data have been presented in a manner which, although unconventional, may help to show (1) the amount of redundant information insuring accuracy throughout most of the structure, (2) the regions where overlaps or redundancy are limited, and (3) the very large fraction (182 residues or 83%) of the structure which was determined by mass spectrometry. In addition, it may be useful to note that the heavy dark line above the sequence indicates the structure also determined unambiguously for the type I CAT specified by plasmid R429. The sole differences detected between these two proteins thus far have been the tighter binding to affinity resins of the enzyme encoded by R429 and the amino acid difference at residue 162, the substitution of Ala for Asn.

Several regions of the structure were particularly troublesome. The N-terminal sequence analysis by Edman degradation using both liquid ('spinning cup') and solid phase techniques gave a sharp drop in signals after Gln 15, probably due to cyclisation and blockage. The crucial chymotryptic peptide spanning the region from His 17 to Phe 25 was in low yield and contained Arg. Although sequenced by the dansyl-Edman approach, it was only correctly placed in the sequence when it became obvious that it was contained in the C-terminal part of the elastase fragment spanning Asp 12 to Ser 27. The 'expected' L4 tryptic peptide (Glu 20 at N-terminus) was never isolated and CB-2 was a low-yield 66-residue fragment which was known only by its analysis, and by N- and C-terminal sequences.

A second region of the structure which gave rise to difficulties until the late stages of the project was the two amino acid overlap at 162–163, determined both by dansyl-Edman and mass spectrometry on the elastase fragment Asn 162 to Val 170.

Our search for ambiguities and errors in overlapping peptides in the regions mentioned was prompted by access to the DNA sequence data of the Basle group²⁶ for the region of their transposon which corresponded to the first 142 amino acids of

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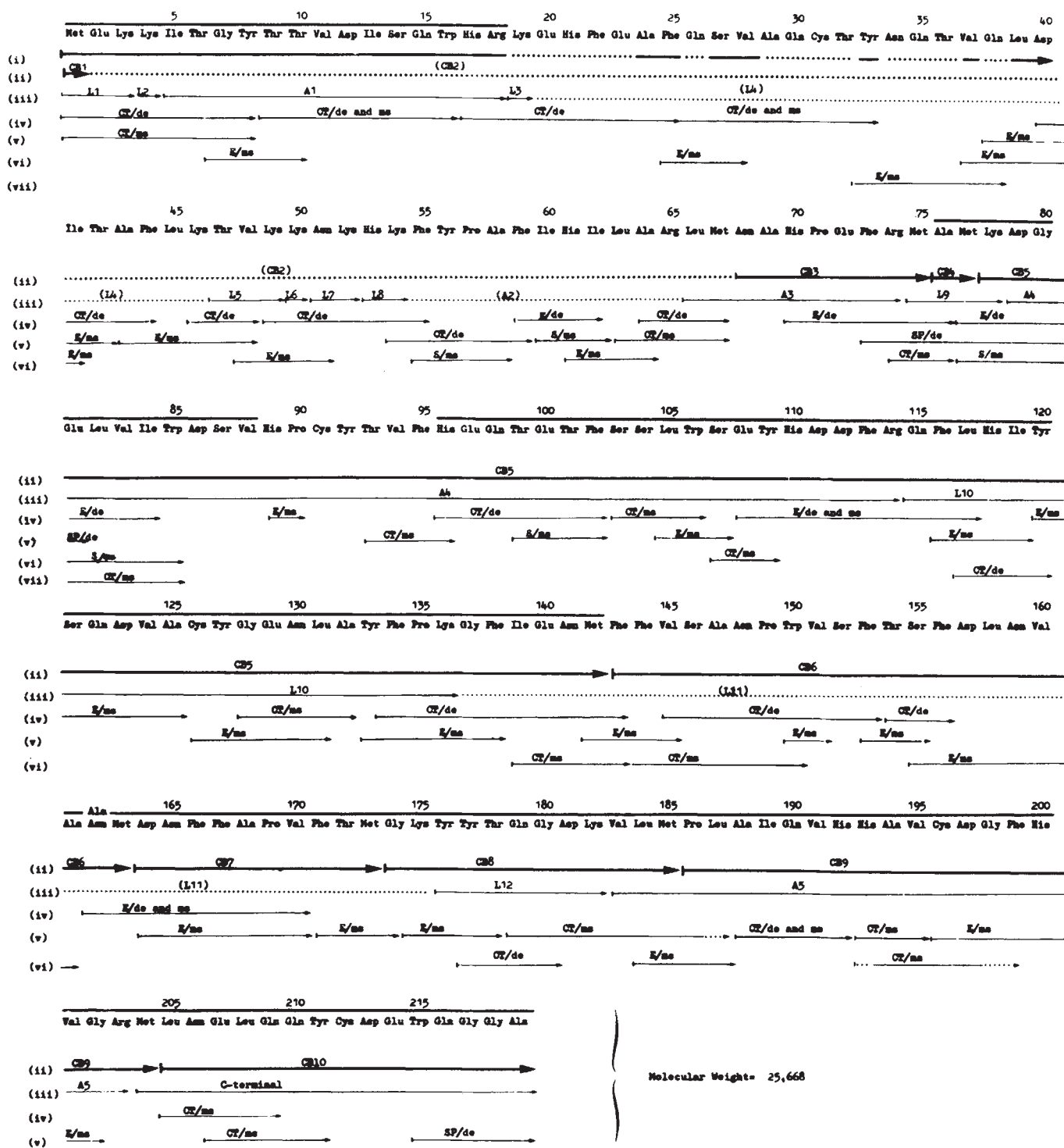


Fig. 1 Amino acid sequence of chloramphenicol acetyltransferase specified by plasmid JR66b. Line (i) indicates the results of automated Edman degradation to residue 40. Dotted underlining indicates ambiguous results or no signal on thin layer, gas chromatography, or back hydrolysis of PTH amino acids. Line (ii) shows cyanogen bromide fragments which were sequenced either by automated Edman degradation or subdigestion with proteases to yield smaller peptides which were sequenced by the dansyl-Edman technique. Line (iii) shows the tryptic fragments and lines (iv)–(vii) indicate peptides generated by digestion of the protein with chymotrypsin (CT), subtilisin (S), elastase (E), or staphylococcal protease (SP) and sequenced by mass spectrometry (ms) or the dansyl-Edman method (de). All cyanogen bromide digests were performed on S -cysteinyll¹⁴C-carboxymethylated protein and the fragments were purified by a combination of gel filtration in 20% formic acid or 6M guanidine hydrochloride and DEAE-cellulose chromatography. One chymotryptic digest was performed on performic acid oxidised protein and one on enzyme which had been S -aminoethylated with ethylene imine. The elastase and staphylococcal protease digests were performed on 14 C-carboxymethylated protein. Peptides from chymotrypsin and elastase digests were purified by Dowex 50 ion-exchange chromatography using a pyridine-acetate eluant or by DEAE-cellulose chromatography with a linear gradient of ammonium bicarbonate. High voltage paper electrophoresis and chromatography were used for purification of pooled column fractions after removal of salt by lyophilisation or gel filtration on Sephadex G-10. The numbering system for tryptic peptides takes into account hypothetical peptides which were never isolated (dotted lines). The same convention was used for cyanogen bromide fragments. The heavy line above the amino acid sequence indicates the extent of sequence analysis performed on the CAT specified by plasmid R429, all of which is identical with that of plasmid JR66b save for Asn 162 which is Ala in the R429 variant of CAT.

CAT. Re-alignment of the peptides in the regions noted above yielded the primary structure shown in Fig. 1.

Although the data summarised in Fig. 1 are presented in such a way as to summarise the evidence from which the structure has been deduced, we have drawn attention to regions which may be of functional importance. In the native enzyme the chloramphenicol binding site seems likely to include portions of the structure near Cys 31 as well as the segment from His 192 to Cys 196 (ref. 19). Studies done on a staphylococcal variant of CAT, which shows a high degree of conservation in the latter region but less than 50% homology elsewhere, have implicated His 192 on the basis of experiments with alkylation by iodoacetamide and diethylpyrocarbonate inhibition¹⁷. The protection afforded by chloramphenicol to inhibition at all three residues is not seen with acetyl-S-CoA. In the fully folded native structure, the coenzyme-binding domain may exist within the N-terminal one-third of the polypeptide. The evidence on this point is both circumstantial and indirect; the truth will be known with certainty on the completion of high resolution X-ray diffraction studies which are in progress²⁰. However, we note that there is a potential adenine nucleotide binding site in the N-terminal one-third of the polypeptide where application of the secondary structure parameters of Chou and Fasman¹⁰ yields a desired domain consisting of alternating β structure and α -helix²².

The matter of the points of contact between subunits of the CAT tetramer has recently been approached in the light of earlier studies on the ease with which very stable hybrid CAT tetramers can be generated *in vivo* and *in vitro*¹². It seemed likely that salt bridges between monomers might be involved and that a search for 'buried' lysine residues might reveal one or more which, especially if found consistently among CAT variants, might be prime candidates for sites of contact between subunit interfaces. The strategy used was an extension of that described previously²³ and involved treatment of the native tetramer with nonradioactive methyl acetimidate followed by removal of the reagent, unfolding of the protein in guanidine hydrochloride and treatment with ¹⁴C-labelled methylacetimidate to label any lysine residue previously shielded from solvent and unlabeled reagent. A unique amino group was modified by the radioactive imidoester, that of Lys 136 (L.C. P., unpublished data).

The amino acid sequences of most proteins—in the absence of further information on secondary or tertiary structures or collateral data on related proteins—are not particularly provocative, and the structure for CAT is no exception. Although much of the primary structures of two other plasmid coded CAT variants are known (*E. coli* type III and *Staphylococcus aureus* type C), an assessment of homology would be premature, apart from that which has already been made concerning the putative chloramphenicol binding site. Similarly, X-ray diffraction studies have not yet proceeded to a stage where useful inferences may be drawn.

The completion of the nucleotide sequence of Tn9 by Alton and Vapnek³ and by the Basle group²⁶ and the rapid progress made by other groups on analogous or homologous chloramphenicol resistance transposons have reinforced the points made by Sutcliffe that DNA sequence analysis offers compelling advantages in terms of time and resources as compared with protein sequencing²⁴. On the other hand, the availability of limited amino acid sequence data on a structural gene of interest may be of considerable assistance as a guide to ambiguities in reading frame and is essential for anticipating post-translational modifications²⁵. The evidence presented in this communication, that of the companion paper³ and that of Marcoli *et al.*²⁶ indicate that in the case of the type I variant of CAT the results of the two approaches give identical results. Further studies on the CAT 'family' of proteins are now likely to proceed more rapidly as nucleotide sequence analysis replaces amino acid sequencing as the primary tool for generating primary structures for evolutionary studies and for the interpretation of electron density maps. However, we wish to stress the continued importance and

necessity of protein chemistry for studies of catalytic function, subunit interactions, and ligand binding.

This work was initiated at the MRC Laboratory of Molecular Biology, Cambridge where W.V.S. and B.D.B. were visiting scientists. Much of the mass spectrometry work was done in the University Chemical Laboratory, Cambridge. We are grateful to both institutions for assistance and support. John Bridgen and John Walker were especially helpful with automated N-terminal sequence analysis. Subsequent studies were supported by the MRC in the form of a project grant (Leicester) and a programme grant (Imperial College). We thank F. Bolivar for base sequence data for the regions flanking the *EcoRI* site (Glu 72/Phe 73), information which first alerted us to an overlap error. We are particularly grateful to T. Bickle, S. Iida and R. Marcoli for early discussions of their proposed structure for the transposon derived from plasmid NR1, and we thank N. K. Alton and D. Vapnek for sharing their data before submission for publication. *Note added in proof:* The primary structure of a transposable element smaller and analogous to that specifying the type I variant of CAT has now been determined²⁶. Knowledge of the amino acid sequence of CAT and the nucleotide sequence of Tn9 is likely to be particularly useful in the light of recent independent experiments by two groups which show that the gene product of Tn9 is expressed well in yeast (*Saccharomyces cerevisiae*) when the chloramphenicol resistance determinant is part of a cointegrate composed of the yeast 2- μ m plasmid and an *E. coli* vector (J. D. Cohen, R. Eccleshall, R. D. Needleman, H. Federoff, B. A. Buchferer and J. Marmur; and C. Lichtenstein and C. Scazzocchio; personal communications to W.V.S.).

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***E. coli* ribosomal RNA contains sequences homologous to insertion sequences IS1 and IS2**

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The insertion sequence (IS) elements, IS1 and IS2, present in multiple copies in the *Escherichia coli* chromosome^{1–3}, are transposable genetic elements of known nucleotide sequence^{4,5}. These elements can modulate gene expression^{6–7}, but it is not known whether they normally function in genetic control. To

determine whether IS elements could exert control through specific RNA transcripts, we hybridised λ NNC₁₈₅₇r14 (carrying IS1) and pBR322 (carrying a portion of IS2) to Northern blots⁸ of *E. coli* RNA. Regions of homology between the IS elements and ribosomal RNA were observed. Computer analysis of reported nucleotide sequences detected large segments of homology between the IS elements and both 23S and 16S rRNA. Additional homologous sequences in Φ X174 and a leader region of a ribosomal protein gene cluster were also detected. The homologous sequence between IS2 and 16S rRNA is the same sequence in Φ X174 DNA which codes for the ends of the *E* and *D* gene and the start of *J*. The partial IS sequences may represent silent evolutionary remnants or they could modulate the expression of genes carrying these sequences.

IS elements are specific segments of DNA which can undergo *recA*-independent translocation between DNA sequences with no apparent homology. These elements can modulate gene expression, cause polar mutations when integrated into proximal regions of operons, and act as vehicles for chromosomal rearrangements^{6,7}. They inactivate genes into which they insert, and reactivate them on precise excision. Two insertion sequence elements, IS1 and IS2, which are present in multiple copies in the *E. coli* chromosome¹⁻³, have been entirely sequenced^{4,5}. IS1 contains several translation stop signals in all three reading frames and causes polar mutations when inserted in either orientation. Integration of IS2 in one orientation can cause

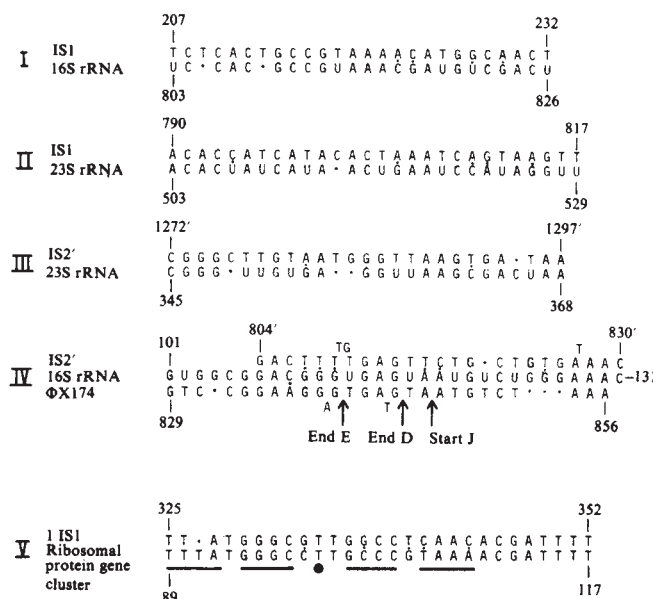


Fig. 2 Nucleotide sequence homologies detected by computer analysis. The computer program described by Korn and coworkers¹³, as modified by Friefeld and Brutlag, was used to compare the nucleotide sequences of IS1 (ref. 4) and IS2 (ref. 5) to a portion of 23S rRNA¹², 16S rRNA¹², Φ X174 DNA¹⁷, and a 3,000-base pair ribosomal protein gene cluster¹⁸. The numbers correspond to the coordinates from the published sequence. Primes correspond to the complementary strand of the reported sequence in inverted order. Dots indicate mismatches. The sequences shown are all considered statistically significant by the criteria and calculations defined by Brutlag.

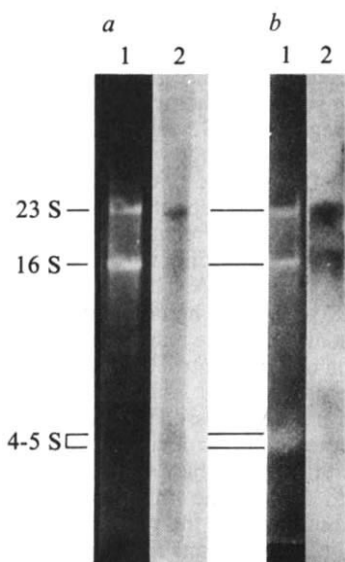


Fig. 1 Hybridisation of IS1 and IS2 to *E. coli* RNA. Whole cell RNA from an RNase⁻ strain of *E. coli* K12 (Miles) was treated for 30 min at 37°C with 10 μ g ml⁻¹ of RNase-free DNase (Worthington) and then inactivated by heating to 65°C for 3 min. The RNA was then separated into its component size classes by 2% agarose gel electrophoresis in 20 mM phosphate buffer, pH 7.5. Ethidium bromide visualisation of two separate experiments are seen in *a* 1 and *b* 1. The RNA in the gels was blotted and bound to treated filter paper as described elsewhere⁴. Wild-type λ and λ NNC₁₈₅₇r14 (IS1) DNA obtained from purified phage particles⁵, and purified plasmid pBR322 and pBR322 (IS2) DNA (described in ref. 9) were nick-translated by the method of Rigby *et al.*²⁰ using ³²P-dCTP (>300 Ci mmol⁻¹, NEN). These probes were hybridised to the above RNA containing filters as described⁹ with the following exceptions: tRNA was used as cold carrier, and the filter paper was washed after hybridisation four times in hybridisation buffer at 37°C for 30 min, then four more times in 0.2 $\times$ SSC, 0.1% SDS at 37°C for 30 min. Wild-type λ DNA and pBR322 (without the IS2 insert) did not hybridise to the filters. Autoradiograms of hybridisation of λ NNC₁₈₅₇r14 (IS1) and pBR322 (IS2) are seen in *1b* and *2b*, respectively. The predominant 23S, 16S and 4-5S rRNA size components are indicated.

initiation of transcription of a proximal gene presumably because the IS sequence contains a promoter¹⁰. Integration of IS2 in the opposite orientation causes transcription termination, possibly due to the presence of a *rho*-sensitive transcription termination signal¹¹. IS2 also contains several translational stop signals in all three reading frames⁵.

There has been speculation concerning the role of IS elements in gene expression and evolution^{6,7}, but it is not clear whether these elements normally function in genetic control. Further, it is not known whether these elements act only at the DNA level (that is, promoter sequences, sequences inducing polarity), or can control gene expression at the post-transcriptional level. If portions of the IS elements exert their function through specific transcripts, then regions of homology between the IS elements and *E. coli* RNA should be detectable. To examine this possibility, labelled probes containing IS1 and IS2 were hybridised to Northern blots⁸ containing total *E. coli* RNA which had been fractionated by electrophoresis on agarose gels. In a previous study, regions of limited homology between IS1 and IS2, not ordinarily observable by solution hybridisation techniques, were readily detected by hybridisation to DNA restriction fragments blotted to nitrocellulose filters⁹. In the present study, total *E. coli* RNA was separated into its predominant 23S, 16S and 4-5S size components by agarose gel electrophoresis (Fig. 1, lanes *a* 1, *b* 1) and chemically bound to filter paper as described by Alwine and coworkers⁸. ³²P-labelled DNA from wild-type bacteriophage λ , λ NNC₁₈₅₇r14 (containing IS1), plasmid pBR322, and pBR322 which contains two-thirds of IS2 plus a small segment of λ DNA was then hybridised to strips of filter paper containing the separated RNA.

The autoradiograms in Fig. 1, lanes *a* 2, and *b* 2, show the result of the hybridisations of λ NNC₁₈₅₇r14 (IS1) and pBR322 (IS2), respectively, to *E. coli* RNA. IS1 hybridised pre-

dominantly to 23S rRNA and to a lesser extent to 16S rRNA. IS2, however, hybridised equally well to both 23S and 16S rRNA. Due to the exceedingly short half life and unique complexity of mRNA, this experimental approach would probably not detect similar sequence homologies with the less abundant messenger RNA fractions which are present on the filter between 16S and 4-5S rRNA. λ and pBR322 probes without the IS elements, isolated in an identical manner to those containing inserts, did not hybridise to the RNA. Furthermore, the purified RNA was treated with DNase before electrophoresis to exclude the possibility of chromosomal DNA contamination.

A large portion of the sequence of the 16S and 23S rRNAs from *E. coli* is known (ref. 12 and C. Squires, personal communication). Therefore, with the aid of a computer program devised by Korn and coworkers¹³ we attempted to find the regions of homology by comparing the nucleotide sequences of the IS elements with that of the RNAs. Sequences homologous to large regions of IS1 and IS2 (greater than 25 base pairs) were found in both 23S and 16S rRNA (Fig. 2). In addition to the sequences shown, numerous shorter regions of homology were detected between the IS elements and the 23S and 16S rRNAs. These shorter sequences were found in clusters in regions which had extensive hyphenated homology. In the case of the 23S rRNA, the regions which can form secondary structure in the RNA (inverted, complementary repeats) involve the same sequences which have hyphenated homology with the IS elements. The regions of IS1 and IS2 found in 23S rRNA (Fig. 2II, III) correspond to the terminal regions of these IS elements.

The portions of the IS elements present in ribosomal RNA can be viewed as nucleotide sequences that have become stably integrated over the course of evolution. The IS elements, or sequences like them, could at one time have interrupted DNA sequences coding for ribosomal RNA; the sequences remaining in the rRNA could represent remnants left behind by imprecise excision or duplication. It is also possible that IS elements which interrupt ribosomal DNA genes are partly processed out at the RNA level.

The partial IS sequences detected in this study may represent regions that have been conserved because of a required role in ribosome function. For example, homologous regions between IS elements and ribosomal RNA could modulate the translation of mRNA carrying IS elements or portions of these elements. Messenger RNA carrying IS sequences could be recognised by the ribosomes by virtue of the sequence homology between the IS elements and 23S and 16S rRNA described here. Hybridisation between the putative IS-mRNA and the ribosomal region containing IS homology could alter an essential RNA-RNA or RNA-protein interaction within a region of extensive secondary structure in the ribosome, making the ribosome either more or less efficient. One consequence of this mechanism would be to protect the cell from insertions which would adversely affect essential functions. This type of mechanism, to circumvent gene inactivation due to IS insertion, predicts that portions of IS sequences should be found in mRNA. We therefore examined other known coding sequences for regions homologous to the insertion elements to determine if regions of partial IS homology resided in specific regulatory sequences. Significant regions of homology were found between IS2 and Φ X174 DNA and IS1 and a ribosomal protein gene cluster, as shown in Fig. 2.

To test whether homologous sequences similar to those shown in Fig. 2 for the IS elements, rRNA, Φ X174 DNA and the ribosomal protein gene cluster could be found between any large DNA or RNA, the nucleotide sequences of IS1, IS2, 23S rRNA, 16S rRNA, the plasmid pBR322 (ref. 14), SV40 DNA¹⁵, and two histone genes¹⁶ were compared with each other to identify regions of homology. The only significant regions of homology found, other than those shown in Fig. 2, were between IS1 and IS2 (ref. 9).

Homology between IS2 and Φ X174¹⁷ detected by computer analysis (Fig. 2, IV) was directly observed when labelled Φ X174 DNA was hybridised to a Southern blot containing IS2 DNA.

The region of the Φ X174 chromosome which is homologous to a 30-base pair nucleotide sequence in IS2 DNA corresponds to the terminus of the overlapping *D* and *E* genes continuing through to the start of the *J* gene. As shown by Sanger and coworkers¹⁷, this sequence contains a ribosome-binding site which is complementary to the 3' end of 16S rRNA. Remarkably, the homologous sequence between Φ X174 DNA and IS2 overlaps the region of homology between IS2 and a region near the 5' terminus of the 16S ribosomal RNA.

The 3,000-nucleotide sequence coding for a ribosomal protein gene cluster¹⁸ includes the genes for ribosomal proteins L11, L1, L10, L7/L12, a portion of the gene for the β subunit of RNA polymerase, and two promoter regions (P_{L11} and P_{β}). A 95-base pair leader sequence before the first ribosomal protein (L11) structural gene contains a 23-base sequence which has dyad symmetry. A nucleotide sequence spanning the entire palindrome in this transcribed leader sequence was found to be homologous to a 29-base sequence in IS1 (Fig. 2V). This homology is especially interesting in light of the observation made by Rak¹⁰, who showed that *gal* RNA isolated from a mutant strain which contained an IS2 insertion in the *gal* operon carried a short nucleotide sequence homologous to IS2 DNA. The orientation of the insertion was such that it could act as a promoter of transcription. Because IS elements contain transcription and translation control sequences, it is possible that the type of homologous sequences found between IS2 and the leader sequence in the ribosomal protein gene cluster represent recognition sites into which IS1 and IS2 can integrate and thus affect gene expression. This idea is supported by the fact that most of the IS1 and IS2 insertions into the *gal* operon occur in the leader sequence¹⁹.

Several observations suggest a correlation between the potential for secondary structure in DNA and RNA and the regions containing sequences homologous to the IS elements: (1) the terminal regions of IS1 and IS2, which are inverted repeats, have sequence homology with rRNA; (2) the sequence homology between IS1 and the ribosomal protein gene cluster involves a palindrome in the leader region of the protein gene cluster; (3) the clusters of hyphenated homology between IS1, IS2 and 23S rRNA correspond to the regions of putative secondary structure in the RNA. This correspondence between the location of IS sequences homologous to rRNA and the presence of secondary structure may have practical significance in the coordination of gene control throughout several levels of the regulatory hierarchy.

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X-ray crystallography of the binding of the bacterial cell wall trisaccharide NAM-NAG-NAM to lysozyme

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Hen egg white lysozyme was the first enzyme whose structure was determined by X-ray crystallography¹. The proposed mechanism²⁻⁴ based on this structure involves the distortion of the saccharide residue (2-acetamido-2-deoxy-D-muramic acid, NAM) in the natural substrate⁵ (an alternating $\beta(1 \rightarrow 4)$ linked oligomer of 2-acetamido-2-deoxy-D-glucose (NAG) and NAM residues) bound to site D in the binding cleft. The importance of substrate distortion has prompted numerous enzymatic⁶, chemical⁷, theoretical⁸⁻¹², and physical¹³ studies, but there is little direct crystallographic evidence on the conformation of a NAM residue bound at site D. We now present the X-ray structure of the non-hydrolysed¹³ trisaccharide NAM-NAG-NAM bound in subsites B, C, D. Our interpretation of the 2.5-Å resolution difference map does not involve distortion of this residue in site D. Comparison with the structure of the δ -lactone derived from tetra *N*-acetylchitotetraose ((NAG)₃NAL) bound to lysozyme¹⁴ suggests we may be looking at a Michaelis complex.

Crystals of native hen egg white lysozyme (Sigma, lot L-6876) were grown by a vapour diffusion technique¹⁵. The crystals of lysozyme with the NAM-NAG-NAM(MGM) trisaccharide bound were grown by co-crystallisation from a solution of 8 mg of MGM and 15 mg lysozyme in 0.5 ml of 4% NaCl buffered with 0.1 M NaOAc at pH 4.5. The native and MGM-bound

lysozyme crystals displayed remarkably different habits thus giving an early indication that MGM had been incorporated into the tetragonal crystals of lysozyme in spite of the relatively small binding constant, $3.6 \times 10^2 \text{ M}^{-1}$ (ref. 13). The unit cell dimensions of the native hen egg white lysozyme crystals are $a = b = 79.08(2)$ and $c = 38.02(1) \text{ Å}$, whereas those for the crystals of MGM bound lysozyme are $78.97(2)$ and $38.25(1) \text{ Å}$.

The structure factor amplitudes for both the native and the enzyme-trisaccharide complex were brought to the absolute scale using the scale factors determined from Wilson-type plots of the corrected intensity data¹⁶. The appropriately scaled data sets thus had an agreement factor of $0.120 (\sum |F_{\text{MGM}}| - |F_{\text{N}}|) / \sum |F_{\text{N}}|$ for 3,652 common reflections. The phases for the reflections of native lysozyme for the calculation of the difference map were those from the refined tetragonal structure which had a conventional R of 0.23 (refined phase angles supplied by Ms D. Grace). Initial examination of a difference mini-map contoured at intervals of 0.03 e Å^{-3} starting with $\pm 0.10 \text{ e Å}^{-3}$ confirmed binding of the substrate MGM to sites B, C and D of lysozyme. Detailed analysis of the map and fitting of the model to the electron density were carried out on an MMS-X interactive graphics system devised by David Barry of Washington University. The model of MGM used for fitting was constructed by docking the atomic coordinates of two crystal structures: $\beta(1 \rightarrow 4)$, α -*N,N'*-diacetylchitobiose (monohydrate) by Mo and Jensen¹⁷ and *N*-acetyl- α -D-muramic acid by Knox and Murthy¹⁸. The atom numbering scheme for MGM is given in Fig. 1a.

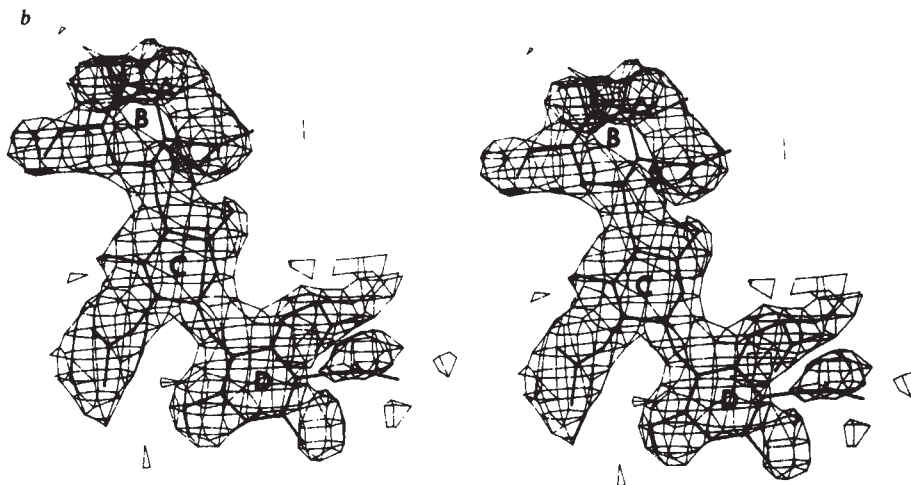
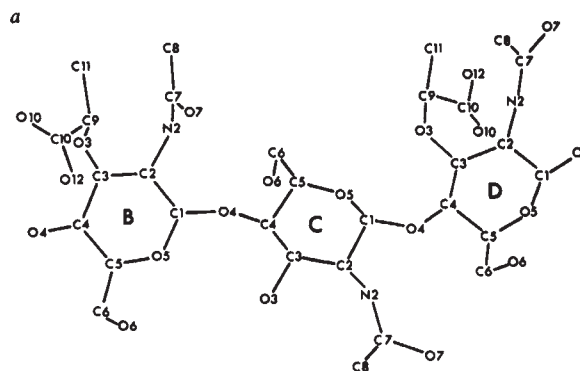


Fig. 1 *a*, The atomic numbering of the molecule of the trisaccharide, NAM-NAG-NAM, that was used in this study. Crystals of the NAM-NAG-NAM bound form of lysozyme were grown by co-crystallisation of MGM and lysozyme. Intensity data were collected from a crystal of native lysozyme and from a crystal of the MGM bound form on a Packer FACS-1 diffractometer¹⁹. Data from 10.0–2.5 Å resolution were measured from two symmetry equivalent (hkl and khl) reflections of each crystal. After correction of each data set for absorption²⁰ (maximum correction 1.31 for the native crystal; 1.51 for the MGM bound crystal), radiation damage²¹ (maximum decay correction at $2\theta = 36^\circ$ was 14% for native and 25% for the substrate bound crystal) and Lorentz-polarisation correction²², the two symmetry equivalent reflections were averaged. The symmetry equivalent agreement indices for the intensity data thus measured are 2.86% for the MGM/lysozyme crystal and 2.30% for the native crystal. *b*, Stereoview of the difference electron density distribution ($|F_{\text{MGM}}| - |F_{\text{N}}|$), α_{N} with the fitted model of NAM-NAG-NAM superimposed. The sugar residues are labelled B, C and D to correspond to the binding sites on the lysozyme molecule¹⁻⁴. This and subsequent figures were produced on an MMS-X interactive graphics system at the University of Alberta.

The model was initially positioned in the electron density by making use of prominent features of the well-defined density associated with the ring in site C, that is, C₆-O₆ and the 2-acetamido group (Fig. 1*b*). In this and all subsequent manipulations of the model into the electron density, only allowed rotations about single bonds were used. Ring puckers were all left in the ⁴C₁ chair conformation for the final fitting procedure. Attempts were made to fit the alternative conformations for the sugar residue in site D, which were discussed by Ford *et al.*¹⁴ into the electron density associated with the trisaccharide MGM. All conformations other than the ⁴C₁ chair for this residue fit less well. In these latter trials, atoms C₁ or O₁ were positioned too far out of the electron density contour surface.

Fitting of the lactyl moieties of both rings B and D was problematic. Simple rotations about permissible bonds produced no better fit for either group. The bond angles at O₃ and C₉ were increased to try to move the lactyl groups into the flattened density. However, no improvement of fit was achieved, so these groups were left with standard bond angles and lengths.

The difference electron density map in the regions of the gluco-pyranose rings in sites B and D is affected by the displacement of several water molecules from the active site on trisaccharide binding. This phenomenon was also observed by Ford *et al.*¹⁴ in the (NAG)₃NAL difference map. Using the coordinates of water molecules associated with lysozyme as deposited in the Brookhaven Data Bank, we find that there is a water position 1.15 Å from C₂ of ring B and a second water position 2.11 Å from C₈ of ring D. Displacement of these two water molecules results in the poor distribution of electron density near these two atoms of MGM. A total of 10 water molecules are probably displaced by MGM upon binding to lysozyme; the distances between nearby atoms of the trisaccharide and these water positions in the native structure vary from 0.8 to 2.1 Å.

A second major feature of the difference map is a concerted shift of the main chain and side chain atoms from Gly 71 to Leu 75, which is relayed from the active site region via a hydrogen bonding network which includes Ser 72 O, Ser 60 O, Asn 59 Oδ1 and Arg 61 N. Concomitant with the binding of trisaccharide, the indole ring of Trp 62 moves towards site B and Trp 63 towards site C into a possible H bonding position with the carbonyl oxygen atom of the 2-acetamido group of NAG. A small shift of Trp 108 away from the substrate binding region is also suggested by this difference map. The magnitude of the main chain movement was obtained by fitting the difference density with the affected residues as a rigid unit on the graphics system. The difference in the coordinates between the native conformation and the shifted position was 1.8 Å. Similar conformational changes in these same regions of the lysozyme molecule have been described²³. The binding of several acetamido monosaccharides have elicited movements of up to

1.0 Å in the region of amino acids 70–76. The shifts described by Perkins *et al.*²³ are, however, considerably smaller than the 1.8 Å movement indicated for those residues as a result of MGM binding.

The conformation of the active site of lysozyme with MGM bound is illustrated in Fig. 2. The most intimate contact between the substrate and the enzyme occurs in site C with the nitrogen of the 2-acetamido group hydrogen bonded to the carbonyl oxygen of Ala 107. Other stabilising hydrogen bonds and close contacts between MGM and lysozyme are listed in Table 1. This table also contains, for comparative purposes, those close contacts between lysozyme and the tetrasaccharide lactone¹⁴. The lactyl side group of the sugar in site B is in close contact with the native position of the guanidinium group of Arg 125 of a neighbouring molecule (related by $\frac{1}{2}-y, \frac{1}{2}+x, \frac{3}{2}+z$). However, there is clear indication in the difference map of the disruption of the intramolecular ion-pair between Asp 119 and Arg 125 which is present in the native lysozyme structure but prevented from forming when MGM is bound. Coordinates for the fitted model of MGM have been deposited with the Brookhaven Data Bank.

Catalysis by hydrolytic enzymes is described most simply as the lowering of the activation energy barrier of the rate limiting step by the enzyme providing a surface complementary to the substrate geometry at the transition state. How this lowering of the activation energy barrier is achieved is the subject of much discussion. It is quite likely that there are a number of factors involved, some more important than others. The lysozyme mechanism has run successively through several of these factors^{3,4,9,10}, and it has been suggested that electrostatic stabilisation is the most important contributor to the rate enhancement^{11,12}. Stabilisation of the positively charged carbonium ion intermediate of the NAM residue in site D of lysozyme is made by the carboxylate ion of Asp 52, with little distortion of the glucopyranose ring in site D away from the ⁴C₁ chair conformation. On the other hand a crystallographic study of the tetrasaccharide lactone, NAG₃NAL, complexed to lysozyme¹⁴ shows that the δ-lactone is in a conformation close to a sofa and in this conformation the ring atoms C1, C2, C4, C5, O5 are coplanar. It would appear that the lactone does mimic the previously proposed⁴ carbonium ion intermediate¹⁴. The measured association constant of $3.3 \times 10^6 \text{ M}^{-1}$ for the lactone is also supportive of this view²⁴.

The relationship of the binding of the transition state analogue¹⁴ (NAG₃NAL) to the binding that we observe for the trisaccharide MGM is shown in Fig. 3. Also included in Fig. 3 are the positions of the two catalytic residues Glu 35 and Asp 52. It is evident from this Fig. 3*a* that the whole trisaccharide penetrates much less deeply into the active site cleft of lysozyme than does NAG₃NAL. The root mean square deviation of the 43 common atoms between these two molecules in their respective binding modes is 1.23 Å (sites B, C and D). The view in Fig. 3*b*

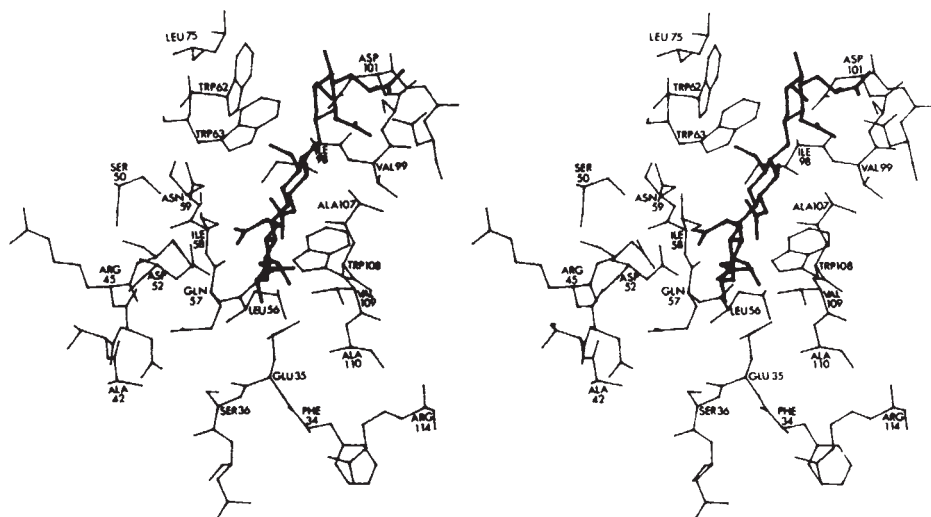


Fig. 2 The active site region of lysozyme with NAM-NAG-NAM bound (heavy lines).

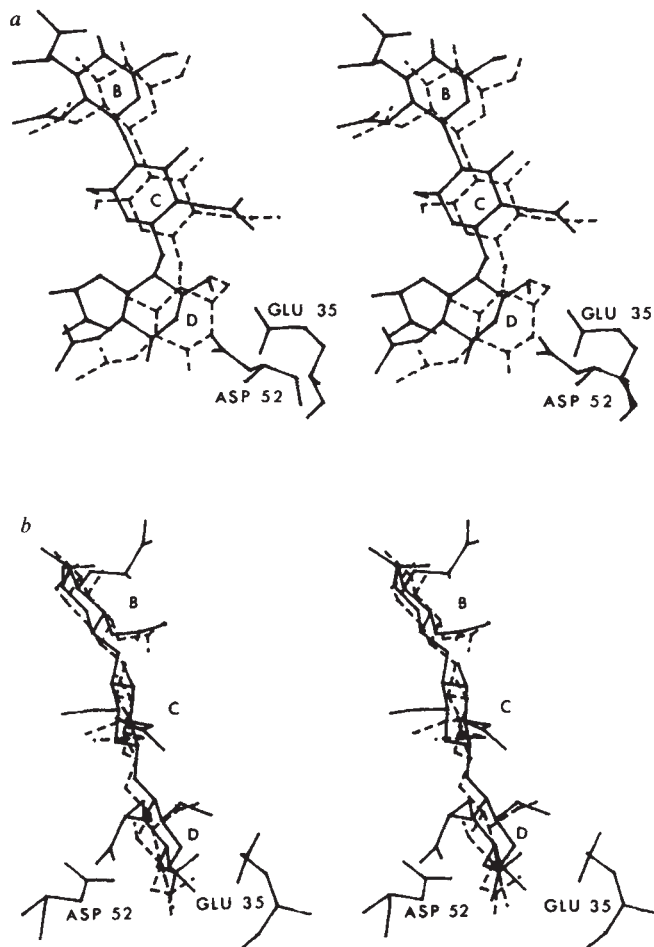


Fig. 3 The difference in the binding modes of MGM (solid lines) and NAG₃NAL (dashed lines) to the active site cleft of lysozyme. Only those residues in sites B, C and D of the tetrasaccharide NAG₃NAL are indicated. The active site residues Glu 35 and Asp 52 have been included to establish the relative orientation of these saccharides to the lysozyme molecule. Comparison of the two orthogonal views *a* and *b* shows that the tetrasaccharide transition state analogue NAG₃NAL penetrates more deeply into the cleft than does MGM. The NAM residue in site D is even further out of the binding site than the lactone ring.

indicates that there is little relative displacement in a direction perpendicular to the opening of the cleft. The NAM residue in site D is even further out of the cleft than the corresponding atoms of the lactone. On this basis we suggest that the binding orientation we observe for MGM may be characteristic of an initial Michaelis complex with the enzyme. Holler *et al.*^{25,26} have observed the formation of stable productive complexes of chitohexose with lysozyme and have suggested that these complexes are subsequently transformed into reactive complexes. It seems clear that the productive binding and conversion to a reactive complex can only be achieved with the additional binding energy provided by the interaction of two more hexose units to sites E and F on lysozyme. Formation of this reactive complex is therefore associated with general acid catalysis by Glu 35 and electrostatic stabilisation¹² of the ensuing transition state carbonium ion by Asp 52.

The mode of binding we observe for MGM is consistent with a number of facts. The association constant determined by Patt *et al.*¹³ is considerably smaller than that of the lactone. Part of this stems from the fact that in the lactone, site A is also occupied by a NAG residue. The remaining differential must stem from the observed differences in the binding modes as described. In the solution NMR study of the binding of MGM to lysozyme¹³, it

was concluded that there was no distortion of the saccharide bound in site D. The coupling constant studied, however, corresponds to the glucopyranose ring of the NAM residue in site D having the α -anomeric configuration at C1 (approximately 66% of the substrate molecules are α (ref. 27)). Binding of the β monomer could not be observed because the chemical shift of

Table 1 Comparison of close contacts between lysozyme and inhibitors*

Site	Protein atom	Substrate atom	Distance with MGM	Distance with NAG ₃ NAL†
B	Asn 103 O δ 1	O 10	2.7	—
	Asn 103 N δ 2	O 10	2.2	—
	Asp 101 O δ 2	O 6	4.7	4.3
C	Ala 107 O	N 2	2.0	3.9
	Trp 108 C δ 2	C 8	3.8	4.0
	Trp 108 C ϵ 2	C 8	3.5	3.2
	Trp 108 N ϵ 1	C 8	2.9	2.6
	Trp 108 C δ 1	C 8	2.7	3.2
	Trp 108 C γ	C 8	3.4	4.2
	Gln 57 O	C 8	3.7	3.6
	Trp 63 N ϵ	O 3	4.0	3.4
	Asn 59 N	O 7	4.2	2.8
	Asp 52 O δ 2	C 5	3.4	2.7
D	Asp 52 O δ 2	O 5	3.5	2.0
	Asp 52 O δ 2	C 1	3.6	2.4
	Val 109 C γ 1	C 2	3.5	3.7
	Val 109 C γ 1	O 5	2.9	4.5
	Val 109 C γ 1	C 1	3.4	4.1
	Val 109 C γ 1	O 6	3.1	3.3
	Val 109 N	O 6	3.4	3.4
	Asn 46 N δ 2	N 2	4.1	3.0
	Asn 46 N δ 2	O 10	3.0	—
	Glu 35 O ϵ 1	O 6	2.8	3.3

* Distances to native coordinates.

† From Ford *et al.*¹⁴.

the β -anomeric proton is near the solvent peak in the spectrum. The α -anomeric configuration requires an axial hydroxyl group. However, there is no electron density which would correspond to an axial hydroxyl group (Fig. 1*b*). Thus, it would appear that the active site of lysozyme in the crystal is capable of preferentially binding the β -anomer in site D. Examination of Fig. 2 shows that if an axial OH were present in the *N*-acetylmuramic acid in site D there would be close contacts with the side chains of Asn 46 and Asp 52 which could be relieved, presumably, by a further rotation of the sugar ring out of its observed binding site.

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Quasi-hexagonal molecular packing in collagen fibrils

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Collagen molecules in native 66.8 nm (*D*) periodic fibrils are widely believed to be assembled into discrete, rope-like sub-structures, or microfibrils¹⁻¹⁷. Several types of microfibril have been proposed (2, 4, 5, 7- and 8-stranded), mainly on the basis of information contained in the medium angle X-ray diffraction patterns of native tendon fibres^{1,5-7}. These patterns show a series of equatorial and near-equatorial Bragg reflections which indicate that the collagen molecules are arranged on a three-dimensional crystalline lattice. The 4-stranded¹¹, 5-stranded^{4,6} and 8-stranded^{7,8} microfibrils are *D*-periodic with approximate diameter 3.8 nm, and these and the 2-stranded¹⁰ model are supposed to be packed on a three-dimensional lattice whose basal unit cell, (approximately) perpendicular to the fibril axis, is tetragonal (or quasi-tetragonal) with side *a*, *a*√2 or 2*a*, where *a* is ~3.8 nm. In this paper we describe a re-interpretation of the X-ray data^{5,6} which leads to a new model for the crystalline regions of the fibril, based on quasi-hexagonal molecular packing without microfibrillar sub-structures, and hence having the character of a molecular crystal¹⁸.

The crystallographic implications of combining hexagonal molecular packing^{1,19-25} with the necessity for intermolecular *n* × *D* staggers^{26,27} have been discussed^{28,29}. Structures with regular *D* (and 4*D*) or 2*D* (and 3*D*) staggers in the three principal directions give rise to several types of crystallographic unit cells²⁹; that suggested by McFarlane²⁸ has Bragg plane separations showing some agreement with the observed X-ray diffraction row line spacings²⁹. However, some strong X-ray reflections are not indexed and, for those that are indexed, the discrepancies between observed and calculated spacings are greater than for the tetragonal indexing schemes. We now show that this type of model can be modified to give a good fit with the

X-ray diffraction pattern. The X-ray pattern shows the most intense Bragg reflections on row line spacings 1/1.369 nm⁻¹, 1/1.328 nm⁻¹ and 1/1.264 nm⁻¹ and, if it is assumed that these three spacings correspond to the principal Bragg plane separations in a slightly distorted (quasi) hexagonal packing scheme (Fig. 1), the agreement between the positions of the row lines predicted by the resulting unit cell and the observed data is excellent (Table 1). The unit cell is *a* = 2.667 nm, *b* = 3.903 nm, $\alpha = 104.58^\circ$ and 17 of the 19 observed spacings are indexed with a standard deviation between observed and calculated reciprocal (or diffraction) spacings¹⁷ of 0.004 nm⁻¹ (compared with 0.010 nm⁻¹ for the tetragonal indexing⁶ of the same spacings). The two other, non-trivial combinations of the three principal quasi-hexagonal spacings give rise to unit cells which do not correspond so well to the observed data. The new unit cell predicts fewer unobserved row lines, in the range of the observed data, than any of the 5.4 nm or larger tetragonal cells. Furthermore, the strong intensity on the three row lines of approximate spacing 1/1.3 nm⁻¹ is a natural consequence of the three principal Bragg plane separations in the quasi-hexagonal lattice.

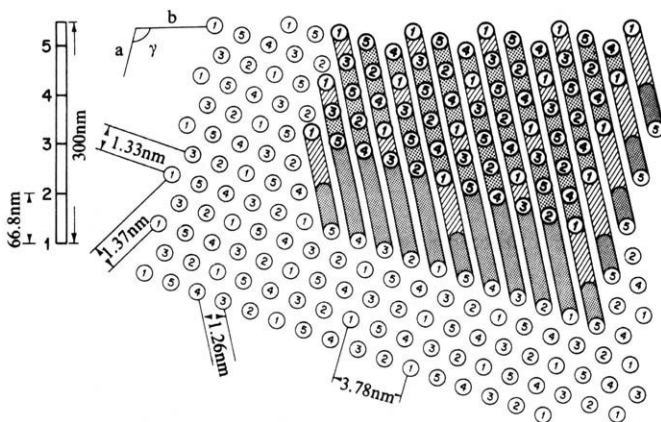


Fig. 1 Possible structure in one region of a collagen fibril, shown in projection down the fibril axis. Numbers refer to molecular segments (see upper left) in one 66.8 nm (*D*) thick transverse section. Thick shaded lines show molecular tilt and connectivity between upper (bold type) and lower (regular type) surfaces of the section. The molecular polarity and sign of the tilt (~5°) are arbitrary. In this example the lateral shift of the unit cell origin in height *D* is $\Delta a = a/10$, $\Delta b = -3b/10$. The same unit cell dimensions could also arise by regular combinations of two 1*D* (and 4*D*) staggers and one 2*D* (and 3*D*) stagger in the three principal directions, as well as by more irregular patterns of intermolecular *n* × *D* staggers.

The fanning of the near-equatorial reflections in the X-ray diffraction pattern indicates that the collagen molecules are inclined at a small angle to the fibril axis, either by tilting of straight molecules or by super coiling⁵. In the present model we propose straight molecular tilting, in such a way that the axes of the molecules lie in the 1.264 nm separated Bragg planes (Fig. 1). The molecules then appear tilted, by about 4°, when viewed perpendicular to the fibril axis along either the 1.369 nm or 1.328 nm separated Bragg planes. The tilting then accounts for the observed intense equatorial 1/1.264 nm⁻¹ reflection and the intense off-equatorial reflections on the 1/1.369 nm⁻¹ and 1/1.328 nm⁻¹ row lines. Since the diffraction pattern arises from a large number of azimuthally disorientated fibrils, the off-equatorial reflections appear symmetrically on either side of the equator.

In general, hexagonal molecular packing of straight tilted molecules with intermolecular *n* × *D* staggers implies a lateral

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shift in the unit cell origin in height D when viewed in projection down the fibril axis. This corresponds to lateral (perpendicular to the fibril axis) shearing of the three-dimensional unit cell and, for a given molecular tilt, the direction and extent of shearing is determined by the unit cell contents. Since lateral shearing of the unit cell in real space (the structure) corresponds to axial shearing (parallel to the meridian) of the reciprocal cell in reciprocal space (the diffraction pattern), reflections on the various row lines will be displaced parallel to the meridian. This effect could account for certain row line reflections indexing as non-integral orders of D , as is observed, for example, on the $1/3.8 \text{ nm}^{-1}$ row line^{5,6}, though more detailed calculations will be necessary to account fully for features such as this. Lateral shearing and molecular tilt are illustrated in Fig. 1.

Table 1 Indexing of observed rat tail tendon row line spacings⁶ with unit cell based on quasi-hexagonal molecular packing

1/observed (nm)	1/calculated (nm)	h	k
3.800	3.778	0	1
2.651	2.582	1	0
2.461	2.436	1	-1
1.890	{ 1.918	1	1
	{ 1.889	0	2
1.748	1.748	1	-2
1.369	1.369	1	2
1.328	1.328	2	-1
1.264	{ 1.264	1	-3
	{ 1.259	0	3
1.224	1.218	2	-2
1.141	1.137	2	1
1.031	1.034	1	3
0.964	{ 0.969	1	-4
	{ 0.959	2	2
0.865	{ 0.870	3	-2
	{ 0.861	3	0
0.815	{ 0.823	1	4
	{ 0.812	3	-3
0.72	0.718	3	2
0.68	{ 0.685	2	4
	{ 0.680	1	5
0.63	{ 0.632	2	-6
	{ 0.630	0	6

Equatorial reflections have been observed on the $1/1.748 \text{ nm}^{-1}$ and $1/0.964 \text{ nm}^{-1}$ row lines. We note that these spacings index as (1, 2) and (1, 4) respectively in the present unit cell (Table 1), and thus correspond to Bragg planes which are almost parallel to the 1.264 nm separated Bragg planes (1, 3). Lattice sampling of the tilted molecular transform on these row lines would therefore be expected to give reflections very close to the equator, and these reflections may appear equatorial as a result of specimen disorientation (1.5°). This model therefore gives explicit account of the equatorial or off-equatorial occurrence of the most intense reflections in the near-equatorial part of the X-ray diffraction pattern.

The preceding discussion has assumed that the row lines are parallel to the meridian of the X-ray pattern. The data from native fibres, however, shows a small angular splitting for several row lines which indicates axial shearing in the structure^{5-7,17}. From the identical angular splittings ($\pm 3^\circ$) of both the meridional and $1/3.8 \text{ nm}^{-1}$ row lines¹⁷, this shearing must be centred about the a axis. These data are only consistent with tetragonal lattices if there is no intensity on two of the four predicted $1/3.8 \text{ nm}^{-1}$ row lines¹⁷, whereas no such problem arises for the quasi-hexagonal model, in which only two $1/3.8 \text{ nm}^{-1}$ row lines are predicted.

Observations in the region of the $1/0.95 \text{ nm}^{-1}$ layer line (from the collagen triple helix) allow the relation between the molecu-

lar tilt and the axial shear to be specified. For native fibres, the intersection of the inner $1/3.8 \text{ nm}^{-1}$ row line with this layer line occurs at a smaller distance from the equator than the intersection of the outer row line. This implies that the row lines are inclined in a direction opposite to that of the molecular tilt, as projected on to the plane of the axial shear. This interpretation is confirmed by the behaviour of these reflections during the so-called orthomorphous transition^{5,17}, in which the angular splitting of the row lines is reduced. The transition is accompanied by a shift towards the equator of both reflections representing the intersections of the $1/3.8 \text{ nm}^{-1}$ row lines with the $1/0.95 \text{ nm}^{-1}$ layer line, as predicted.

Density measurements offer a possible method of discriminating between the different molecular packing models²⁹. These data are somewhat confused, however, by the relative insensitivity of the high angle equatorial X-ray spacing to the water content, in the region of $1/1.3 \text{ nm}^{-1}$ (that is, when the 3.8 nm lattice is observed)^{30,31}. Since it is difficult to imagine how water could enter the fibril without altering the equatorial X-ray spacing this observation has been interpreted as representing an increase in the interfibrillar water content³⁰. Thus, taking a lower limit for the water content at an equatorial spacing of $1/1.31 \text{ nm}^{-1}$ (that is, the centre of the '1.3 nm triplet') of $0.6 \text{ g water per g collagen}$ ³¹, and assuming an average dry collagen density^{30,32,33} and intrafibrillar water density of 1.36 g cm^{-3} and 1 g cm^{-3} respectively, the proportion of the intrafibrillar volume occupied by collagen in native rat tail tendon fibrils is approximately 55%. Table 2 shows that the relative exclusion volumes of the various packing models are distributed over a wide range, with the (quasi) hexagonal models showing the best agreement with the observed data. Independent support for hexagonal packing has been obtained using a molecular probe technique²⁴.

Table 2 Relative exclusion volumes for the different packing models

Basal unit cell			Model	Volume of fibril occupied by collagen
a (nm)	b (nm)	γ (deg)		
3.847	3.847	90	Tetragonal packing of 5-stranded microfibrils ⁶	37%
5.440	5.440	90		37%
7.694	7.694	90		37%
7.56	7.55	90	Tetragonal packing of 5-stranded microfibrils ¹⁷	38%
2.7	4.12	109	Hexagonal packing of molecules ²⁷	57%*
2.667	3.908	104.56	Quasi-hexagonal packing of molecules	54%
2.62	4.02	109	Hexagonal packing of molecules ²⁸	55%*
5.5	5.5	90	Tetragonal packing of molecules ¹⁸ or 2-stranded microfibrils ¹⁰	72%*
3.49	3.84	90	Tetragonal packing of 8-stranded microfibrils ^{7,9}	82%*

* Assuming the c axis is perpendicular to the a - b plane and equal to $n \times 66.8 \text{ nm}$.

It should be emphasised that here we account only for the positions of the sharp Bragg reflections in the near-equatorial part of the X-ray diffraction pattern. The breadth of these reflections measured in a direction parallel to the equator indicates that the lateral extent of long-range order is $\sim 100 \text{ nm}$ (ref. 5). However, at least 50% of the intensity scattered by the specimen in the near-equatorial region lies between the Bragg reflections in the form of continuous diffuse scatter. The profile of this diffuse scatter³⁹ falls sharply away from the origin and

reaches a broad maximum at about $1/1.3 \text{ nm}^{-1}$. Since fibril diameters in adult rat tail tendon are in the range 8–550 nm (ref. 34), it may be concluded that some fibrils or parts of fibrils are crystalline and other fibrils or parts of fibrils are disordered. Alternatively the disorder may exist within the crystalline lattice itself. As the Bragg reflections show very little increase in breadth (as measured parallel to the equator) with increase in distance from the origin, this and the form of the diffuse scatter are indicative of a mixed crystal³⁵—a crystal in which the crystal lattice is well developed but throughout which the unit cell contents vary. Variation in the azimuthal positions of the molecules in the unit cells would cause diffuse scattering. To determine whether this variation is static or dynamic requires measurement of inelastically scattered radiation in the Rayleigh or Brillouin regions³⁶ or measurements of nuclear magnetic resonance³⁷ which already have been interpreted as suggesting dynamic azimuthal movement of the molecules in wet tendon.

Therefore, based on a new interpretation of the X-ray diffraction patterns, we propose that, in the collagen fibrils of tendon, the molecules are tilted to the fibril axis and packed on a quasi-hexagonal lattice that is both laterally and axially sheared. Discrete, rope-like, microfibrillar sub-structures within the fibril are not required by the X-ray data, though the unit cell does contain the minimum *D*-periodic descriptive unit³⁸. The data do not preclude specific cross-linking patterns which may define minimum structural units^{3,11}.

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X-ray diffraction from the side-by-side model of DNA

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An intriguing topological problem posed by the double-helical Watson-Crick¹ model of DNA is that of unwinding the inter-twined strands during replication. Several workers have recently proposed novel side-by-side (SBS) structures for DNA^{2–6}. In all these models the two strands are joined by complementary Watson-Crick base pairs and the antiparallel polynucleotide strands alternate between short segments of right- and left-handed helix, thus both reducing the amount of intertwining and alleviating the unwinding problem. We show here that there are unacceptable discrepancies between the observed diffraction pattern of B-DNA⁷ and that calculated for the original SBS structure². We also describe a simple modification of this model which resolves some of the more serious discrepancies. However, the agreement is still markedly inferior to that obtained for a Watson-Crick model of DNA.

The model of Rodley *et al.*² is the only SBS structure for which detailed coordinates have been published. This model can be considered as most similar to the B conformation of DNA and Rodley *et al.* claim that it can account for patterns of the B type. The B conformation has been observed in both crystalline fibres of lithium (LiDNA) and semicrystalline fibres of sodium DNA (NaDNA). After allowance has been made for the effects of molecular packing, the X-ray scattering from a DNA molecule in NaDNA is, within the limits of the data from these fibres, essentially identical to that from one in LiDNA, indicating a very similar if not identical molecular structure. There is, therefore, a compelling requirement that any SBS model be capable of accounting not only for the data from semicrystalline patterns but also the more extensive and precise data from crystalline fibres.

Rodley *et al.* proposed their model as typical of a whole family of SBS structures, the members of which might differ, for example, in the number of nucleotide pairs in the right- and left-handed regions. However, the crystalline B X-ray data are compatible with the orthorhombic space group $P2_12_12_1$. The symmetry elements of this group when combined with the non-space group systematic absences (when $h+k$ is odd and $l=3n$, where n is integral) indicate that each DNA molecule must contain at least one diad axis per five nucleotide pairs perpendicular to the helix axis and orientated along the *a* or *b* axis. This extra symmetry element requires that the number of nucleotide pairs in the right- and left-handed segments be equal.

In contrast to the claim by Rodley *et al.*, the value of the reliability index (for the agreement between the SBS model and the observed diffraction data) calculated by Arnott⁸ suggests that the SBS model is in substantially worse agreement with observed data than the best model of the Watson-Crick type. It is not clear that a single parameter such as the reliability index is the best way of comparing models of this kind and we compare below the full molecular Fourier transforms of the SBS and Watson-Crick models. This approach allows the regions of the diffraction pattern where the most significant discrepancies occur to be identified and provides an essential basis for any consideration of possible structural refinement of the SBS model.

The model of Rodley *et al.* does not completely solve the unwinding problem as it is still helical. With a minimal distortion of the original coordinates, the structure may be treated as a 10-fold right-handed helix with 10 nucleotide pairs per residue and a pitch of 345 Å. There is 2-fold rotational symmetry within each residue so that the structural asymmetric unit consists of

five nucleotide pairs. The diffraction from such a structure is confined to layer planes with spacing $1/345 \text{ \AA}^{-1}$. Both the method of calculating the Fourier transform⁹ and the correction for the effect of water in the fibre⁷ have been described elsewhere.

A detailed comparison of the diffraction calculated for the SBS model proposed by Rodley *et al.* and that observed for the B form is illustrated in Figs 1 and 3. The diffraction pattern in Fig. 1 is in very close agreement with unpublished calculations of D. R. Davies (personal communication). It is immediately clear that this particular SBS model cannot account for the X-ray diffraction from crystalline fibres of LiDNA. This is because the translational repeat along the helix axis for the SBS model is 345 Å, whereas that required by the X-ray data is 34.5 Å (the value is actually 33.7 Å but this difference is not relevant to the argument). The only possibility for removing this major discrepancy would lie in the extremely unlikely event of a model of this type having a Fourier transform on all layer planes for which $l \neq 10p$ (where p is any integer, including zero) which was accidentally zero. Figure 1 shows that such a fortuitous occurrence is not the case for the published model coordinates.

The translational repeat of the SBS model would, however, be reduced to 34.5 Å (and the predicted diffraction thus confined

only to layer planes which are actually observed) if the rotation per residue were 0° instead of 36° . A model with no net rotation may be thought to be biologically more attractive as the polynucleotide chains do not then intertwine at all. We have, therefore, applied a uniform distortion to the published SBS model to reduce the net rotation to 0° . This was achieved by reducing the atomic ϕ coordinates within the q th nucleotide of the repeating unit by $\frac{25}{10} \times (q-1)^\circ$. This is a crude way of modifying the coordinates but it is quite satisfactory for examination of the calculated diffraction expected from this sort of model. The modified model can be regarded as a helical structure with one residue per helix pitch. As for the original SBS model, the residue consists of 10 nucleotide pairs with 2-fold symmetry. The transform of this model is plotted in Fig. 2.

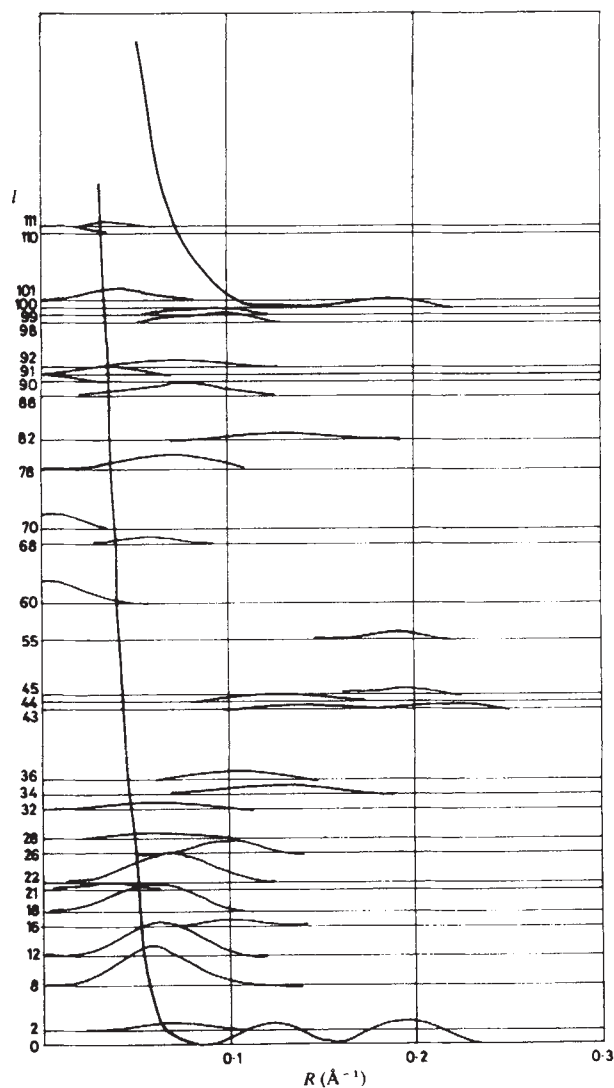


Fig. 1 Cylindrically averaged squared Fourier transform for the side-by-side model for DNA proposed by Rodley *et al.*². The transform on each layer plane l is plotted as a function of the distance R from the centre of the plane.

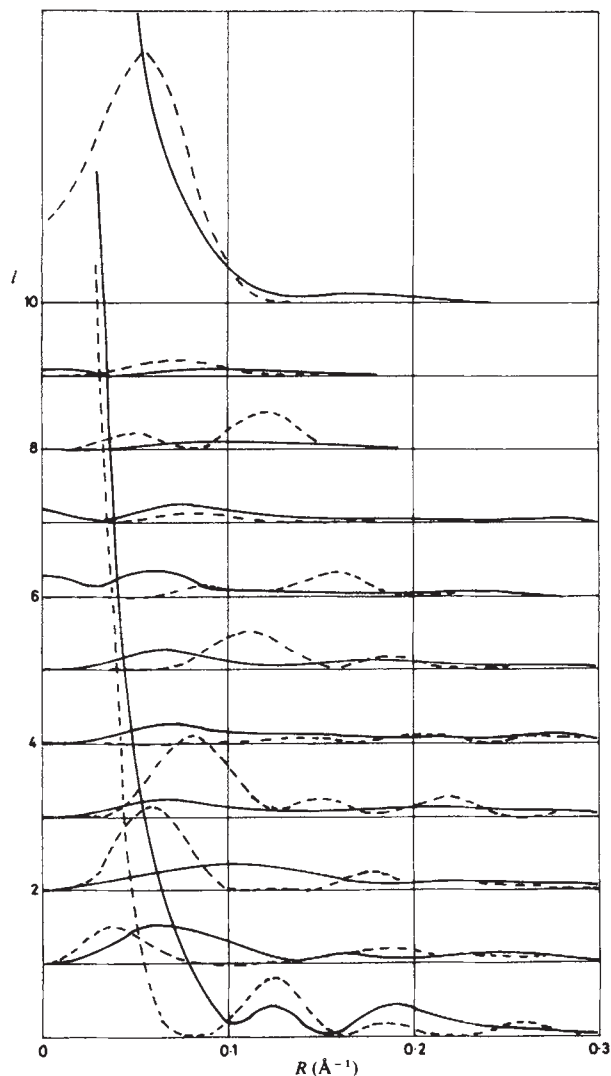


Fig. 2 Comparison of the cylindrically averaged squared Fourier transform of a distorted simplified version of the original side-by-side model (solid curve) with that of the Watson and Crick model for DNA described by Arnott and Hukins¹⁰ (broken curve).

Figure 3 represents the level of agreement which was obtained between observed and calculated diffraction in the initial refinement of the Watson-Crick structure of crystalline LiDNA. The dashed curves in Fig. 2 represent the calculated diffraction for a further refinement of this structure using the linked-atom least-squares technique¹⁰. While there are significant differences between the diffraction calculated for these two

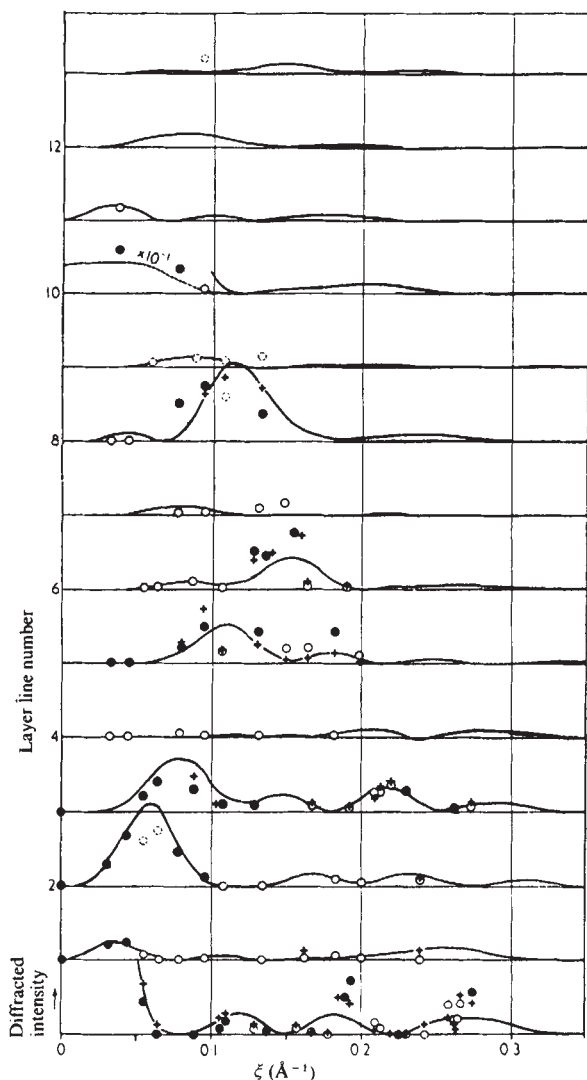


Fig. 3 Comparison of the cylindrically averaged squared Fourier transform of the Watson and Crick model for DNA refined by Langridge *et al.*⁷ with the observed intensities. Observed intensities in order of decreasing reliability are indicated by ●, ○ and ×. ξ is the distance from the centre of the layer line and is equivalent to R in Figs 1 and 2.

Watson and Crick models, the differences are not large and for both models the calculated diffraction is similar to that observed. Even the most superficial examination of Figs 1–3 reveals that the diffraction calculated for the two SBS models differs markedly from that observed.

A particularly serious deficiency of the original SBS model is the occurrence of diffraction on layer planes which are neither observed nor predicted by the Watson–Crick models, that is on $l = 8, 12, 18, 22, 26, 78$ and 88 in Fig. 1. There is no doubt that the quality of the X-ray diffraction patterns from crystalline fibres of LiDNA is sufficient for the original SBS model to be eliminated on the basis of the non-observation of these layer planes. The SBS model also predicts substantial meridional diffraction on layer planes $l = 60, 70$ and 90 (corresponding to $l = 6, 7$ and 9 in the normal B-DNA nomenclature). This is also at variance with the observed diffraction. In addition to predicting diffraction which is not observed, the SBS model fails to predict the strong diffraction which is observed on layer planes $l = 10, 20, 30, 50, 60$ and 80 ($l = 1, 2, 3, 5, 6$ and 8 in the normal nomenclature).

It can be seen from Figs 2 and 3 that while the distorted SBS model only predicts intensity on those layer planes for which

diffraction is actually observed, the magnitudes of these predictions are seriously in error. Specifically, substantial meridional diffraction is predicted but not observed on layer planes $l = 6, 7$ and 9 . Furthermore, the distorted model is seriously in error in its prediction of the relative intensity of diffraction on the various layer planes. These discrepancies are particularly serious on $l = 2, 3$ and 8 where the overall calculated intensity is much less than is observed and on $l = 4$ for which substantial diffraction is predicted where the observed intensity is essentially zero.

We may conclude that there are major and quite unacceptable discrepancies between the observed diffraction from the B form of DNA and that calculated for the SBS model proposed by Rodley *et al.*². Although some of the more serious discrepancies can be removed by the simple distortion of the original model described here, the degree of agreement between observed and calculated diffraction is still very poor and very much inferior to that reported for the best models of the Watson–Crick type. It should also be emphasised that DNA can change reversibly within a fibre from a conformation that gives the semicrystalline B pattern to one that gives a fully crystalline A pattern¹¹. This change is easily explained in terms of the Watson–Crick model by an increase in the number of nucleotide pairs per pitch from 10 to 11. Even if an SBS model could be found to account for the B diffraction data, it would also need to be capable of undergoing a stereochemically plausible transition to account for the A diffraction pattern.

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Errata

The authorship of the article 'Crust of oceanic affinity in Iceland', *Nature* **281**, 347 (4 October) emerged incorrectly as a result of a misunderstanding between us and our typesetters on the telephone. It should have read:

Scientific Party, Iceland Research Drilling Project (I. L. Gibson, editor).

We regret this error.

In the letter 'Chlorophyll and nitrate fine structure in the southeastern Bering Sea shelf break front' by R. L. Iverson *et al.*, *Nature* **281**, 664–666, the units mentioned in the legends to Figs 3 and 5 should read: ($\mu\text{g atoms NO}_3\text{-N l}^{-1}$).

In the obituary to Dr Scott Mazzur, *Nature* **280**, 708, the new antigenic marker mentioned in the penultimate paragraph is the 'I' antigen, not the 'e' antigen as shown.

reviews

Longing for simplicity

P. D. Moore

Plant Strategies and Vegetation Processes. By J. P. Grime. Pp. 222. (Wiley: Chichester, UK, and New York, 1979.) £11.50.

If there is one straw for which all biologists grasp, it is a clear conceptual framework upon which to hang the varied observations which form the raw material of biological research. No biological mind has equalled that of Darwin when it comes to the discovery of common denominators within disparate facts, but many have used a similar approach with varying degrees of success. In a science in which laws are statistical and exceptions are often commoner than the rule, it takes a brave man to make firm statements concerning the way in which living organisms are structured or behave in relation to their environment. That such statements are readily latched on to by the academic masses is evidenced by the current popularity of such concepts as *r* and *K* selection, or island biogeographic theory. We all long for simplicity in nature and embrace it firmly when it is shown to us.

J. P. Grime of the University of Sheffield is one of those people who has actively sought simple statements which can be made about nature and in particular about the various strategies which have evolved within the Plant Kingdom and which confer certain advantages upon those species possessing them. He conceives of three basic strategists: competitors, stress tolerators, and ruderals. He defines competition as the tendency of neighbouring plants to make use of the same resources; the competitor strategist is thus a species which, as a consequence of robust stature, fast growth rate and optimal timing of canopy expansion, is well fitted to obtain at least its fair share of those resources. Stress is defined as the external constraint upon the growth of part or all of the plant; the stress tolerator is thus a species which can continue its growth in conditions which might be expected to curtail the growth of others. Ruderal species are characteristic of disturbed habitats, where disturbance is defined as an interruption to plant biomass accretion. On the basis of this view of plant species, Grime proposes that three selective forces, *C* selection, *S* selection and *R* selection, are operative which had led to the evolution of the observed strategies. In attempting to reconcile this system with the currently prevailing *r-K* selective spectrum, Grime proposes that *R* selection is equivalent to *r*

selection, *S* selection to *K* selection and that *C* selection occupies the intermediate part of the continuum.

Having three, rather than two, defined endpoints in a classificatory scheme means that a simple one-dimensional ordination of species along a line is no longer appropriate and that, instead, one must think in triangular terms (an analogous model to the triangle of texture in pedology). In this way we can admit such bastard strategists as 'stress-tolerant ruderals' and even 'C-S-R strategists' which combine some features of all strategy types.

This system Grime regards as appropriate for any consideration of plants in their established phase. Upon it one must superimpose a further set of subdivisions based upon regenerative strategies, such as vegetative spread, soil seed banks, and so on. Having thus defined and explained his terms, the author then proceeds in the second part of the book to put them to the test, applying them to a consideration of various features and processes found in vegetation, such as dominance, coexistence and succession.

The real test of the value of this novel concept of vegetation is, of course, whether it assists in the explanation and understanding of observed phenomena. In this respect there are many pleasing features about Grime's model. One of its most successful applications, to my mind, is in the explanation of the high species density found in calcareous grassland, where low phosphorus and nitrogen availability can be regarded as a stress which limits the development of *C* strategists and permits the proliferation of *S* strategists and various non-competitive intermediate types.

Large scale magnetic fields

Cosmical Magnetic Fields. By E. N. Parker. Pp. 841. (Clarendon/Oxford University Press: Oxford, 1979.) £45.

ALTHOUGH the same basic physical principles apply to cosmical magnetised plasmas as to those produced in the laboratory, very significant differences arise in their application. Trapping of plasma in the laboratory is carried out with magnetic fields anchored to rigid conductors, and the aim is to produce equilibrium configurations. Astrophysical

Perhaps the least satisfactory aspect of the system is the loose definition of stress. The problem here is that what represents a stress for one species may be beneficial for another (take water-logging, for example). As most ecologists think of species as possessing bell shaped performance curves along environmental gradients, stress really has to be defined separately for each taxon, in terms of its optimum and performance limits. It cannot be defined in general terms.

Grime claims that his model is consistent with Odum's suggestion that the greatest diversity occurs in the middle range of a physical gradient, but this is not so. Odum considered that where conditions were least extreme, diversity would be highest. Grime claims that in such locations the *C* strategists would have a hey-day and plant species density would be low. Higher diversities would thus be achieved as one moves out from the centre towards physical extremes (there must be a better expression than 'stress-gradient' for these axes). In this respect Grime's view seems to accord with reality more closely than Odum's.

This book is stimulating, refreshingly novel, controversial, perhaps a little oversimplified in places, but has one most valuable feature: it forces the reader's mind along new paths. He may not always completely agree with the author's views and conclusions, but he is sure to benefit from examining them. This freshness of approach, coupled with a concise and lucid style make this book a valuable tool for teachers of plant ecology. □

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fields are trapped by gas, however, and gravitational forces give rise to magnetic buoyancy: dynamical activity seems to be the norm, rather than stable equilibrium, because of this absence of anchoring. These magnetic fields play important roles in determining conditions throughout the Universe, as they control the production and flow of the cosmic radiation, affect the accumulation of interstellar gas into clouds, and determine the radiation intensity of bremsstrahlung sources. It is clear that theoretical work would have been aimed entirely at understanding equilibrium configurations of magnetised plasmas in the cosmos if spatially unresolved sources had been the only ones

which could be studied. Observations of the Earth's magnetosphere and of the Sun demonstrate clearly that such studies are, however, too restricted: examination of these objects shows changes in magnetic structure in progress on a whole range of timescales which includes the 22 year magnetic cycle of the Sun and flares lasting minutes. These observations have provided an invaluable stimulus to the theoretician, though it is unfortunately the case that the changes observed are usually so complicated that it is difficult to decide which particular processes cause them.

Although many papers present detailed treatments, books on this rapidly developing subject have tended to illustrate the application of magnetohydrodynamics by treating the basic concepts only. Thus, they have tended to provide brief idealised models of the solar dynamo, solar flares, sunspots, and the Earth's magnetosphere. Such treatments have been welcome because they provide insight into the basic problems, but it is necessary for workers to understand magnetic configurations more fully if they are to explain detailed changes in the radiation intensity from pulsar magnetospheres, accretion disks, supernova remnants, and so on. This book by Professor Parker is therefore invaluable because it illustrates clearly how largescale magnetic fields develop.

As mentioned earlier, observations of magnetic configurations on the Sun have stimulated theoretical work. Parker has been in the forefront of this work on interpretation for many years and his papers provide considerable insight about what is happening at various features. This book now provides a more systematic discussion with a series of chapters explaining the various configurations and processes. It must be stressed, however, that the author points out the relevance to non-solar studies, where applicable, and there are chapters devoted to magnetic fields of planets, and the field of the Galaxy.

The book begins with a chapter on the role of magnetic fields in the astronomical universe. This is followed by chapters on the nature of magnetic fields, the basic equations, magnetic stress and energy, and so on. One striking feature observed in high resolution photographs of the Sun is the non-uniformity of the atmospheric plasma, which generally shows a filamentary character. This reflects the presence of flux tubes as opposed to an 'equilibrium', large scale, continuous distribution treated in most theoretical work. Parker examines the formation of these tubes, their stability, and twisting of the structures. Magnetic buoyancy of the tubes and the activity which arises at the solar surface (and even within galactic structure) due to the absence of anchor points are also discussed.

Considerable attention has been given over the years to magnetic configurations

where rapid relaxation of stress produces solar flares. This topic is discussed in chapter 15, but Parker also considers magnetic reconnection in any general topology where non-equilibrium fields have formed. The diffusion of flux through fluids of high electric-conductivity was once regarded as too slow for reconnection to be significant, but this has had to be reconsidered following laboratory studies of instabilities.

There are also chapters (18 and 19) dealing with formation of magnetic flux, which is a continuing process, as the primordial field has not remained trapped with many of the regions which are accessible to observation.

Parker states in the preface that he intended individual sections to be intelligible without too much reference to earlier chapters. To this end, he begins most chapters with a concise and well-referenced discussion which places the particular problem in context. This is followed by a detailed analysis, and the chapter is terminated with a descriptive summary outlining the application of the

work. Anyone wishing to follow the calculations will certainly require considerable knowledge of the techniques normally used in the field, but the assumptions made, and the conclusions reached, are set out qualitatively in a clear, readily assimilated form. It is hardly the case, as the blurb states, that the non-scientist will find the material readable.

There has been a general reluctance to use SI units in astronomy, but the use of practical units in electromagnetism makes the SI system extremely attractive. In fact, the younger generation of scientists (in British Universities and schools, at least) are not familiar with gaussian units. The reviewer suspects that the adoption of SI by an author of Professor Parker's standing could have accelerated its adoption considerably. He is disappointed that the opportunity has been missed (while recognising that others will disagree), but regards the book as a valuable and stimulating contribution. **W.M. Glencross**

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Social behaviour in insects

Social Insects. Vol. I. Edited by Henry R. Hermann. Pp. 437. (Academic: New York and London, 1979.) \$36; £23.40.

GIVEN the role that the social insects (termites, ants, social bees and wasps) have played in the generation of sociobiological theory, a multivolume treatise which summarises our state of knowledge is indeed a welcome addition to the literature. This is the first of three multi-authored volumes. Volume 3 will be devoted to particular groups of insects, with chapters on honey bees, army ants, and so on. Volume 2 and this volume are more heterogeneous in content, with 2 covering communication, general reproductive behaviour, defensive mechanisms, pre-social insects, social spiders, and the systematics of social Hymenoptera and Isoptera.

The present volume consists of eight chapters, held together it seems only by the fact they all deal with social insects. The authors represent not only a wide array of data, but an incredible diversity of opinion on certain key areas, an example being the 'colony as superorganism' concept. I will return to this later.

Chapter 1 (by H.R. Hermann) serves to introduce the series. Hermann provides a brief review of animal behaviour studies and of the various social insects. It is suggested that studies of the genesis of insect social behaviour may be lumped into three categories . . . termed 'genetic-behavioral', 'serial-behavioral' and 'anatomical'. The 'genetic-behavioral' approach refers to the kin-selection theory of W.D. Hamilton. 'Serial-behavioral' and 'anatomical' include phylogenetic

sequences for the generation of highly social from less social forms. Here are discussed the excellent works of H.E. Evans and C.D. Michener. Although I found it hard to follow all the arguments, the section should be a good introduction to this literature. The conclusion which emerges? I quote from page 16: 'Based on the various hypotheses summarized above, phylogeneticists are in obvious disagreement about phyletic associations and the sequence of events that led to a eusocial state in hymenopterous groups'. I quote this to emphasise also the word hymenopterous. Isoptera receive barely a mention in this chapter. The chapter concludes with a review of various social traits, such as social dominance and territoriality.

Hamilton's kin-selection theory has been the major stimulation to recent work on the social insects, and this volume includes not just one, but two chapters devoted to genetical approaches to social behaviour. C.K. Starr is more biological, while R.H. Crozier goes more into the formal population genetics. Both chapters pretty much ignore the termites, in favour of the Hymenoptera, with their exotic male haploid (haplodiploid) method of sex determination. There is much overlap in the two chapters but this is a benefit as kin-selection theory is not so well defined as the popular literature would suggest. Both chapters provide good reviews of possible pathways for the evolution of helping or altruistic behaviour. Both provide discussions of haplodiploidy and its possible implications for eusocial behaviour. Crozier explains that haplodiploid population genetics is essentially the population genetics of sex-linked genes and discusses the implications of this for a variety of genetical problems.

These two chapters also treat R.D.

Alexander's "parental manipulation" theory for the origin of eusocial behaviour, but they both make the mechanism seem less likely or more difficult to achieve than is really necessary (see Charnov, *J. theor. Biol.* **75**, 451, 1978, for discussion). Finally, both chapters discuss the recent work of Robert Trivers and Hope Hare on sex ratio evolution within eusocial societies. Crozier's discussion is more detailed, with development of theory and plots of original data. Starr's discussion of the major prediction of the Trivers-Hare theory is incorrect. At the bottom of page 58, he lays out the conditions for the worker's equilibrium investment ratio to be 3:1. The statement should be that the workers lay none of the male eggs, rather than all of them, as he claims. These discussions of the Trivers-Hare theory should be supplemented with readings from more recent literature, omitted from this volume. I suggest particularly Alexander and Sherman (*Science*, **196**, 494; 1977) and Evens (*Bioscience*, **27**, 613; 1977). I found both these chapters quite thought provoking and it is nice to have all this 'genetical stuff' in one place. It should greatly facilitate the intellectual battles which make sociobiology so much fun: I challenge any first year graduate student to resolve the apparent theoretical conflict of Scudo and Ghiselin versus Levitt (see Table 5, page 266 in Crozier).

A short chapter by Carpenter and Hermann discusses the fossil evidence on the social insects. C. Baroni Urbani has contributed a very interesting chapter on applications of the concept of territoriality (a defended area in addition to the nest) to social insects. There seems to be good positive evidence for colony level territoriality in termites and ants. In ants much of the active defense is over food sources, so the territory becomes a defense of certain situations rather than a defense of a certain space or area. Territoriality seems to be rare in bees and wasps, with the exception perhaps of males and their mate search behaviour. Recent work by Johnson and Hubbel (*Ecology*, **55**, 120; 1974), however, has shown defense of food sources by stingless bees (not cited by the author).

G.C. Wheeler and J. Wheeler discuss their several (about five) decades of research into larvae of social Hymenoptera. The chapter contains detailed morphological descriptions and some astute suggestions as to the adaptive significance of various structures. They conclude with discussion on the role of larvae in the colony life. I was also charmed by G.C. Wheeler's following disclaimer "At this point I had better make my customary and necessary disclaimer: I am not related to William Morton Wheeler (the great ant biologist) — that is, not genetically. We were, however, closely related academically. G.C. Wheeler is close to defining a "cultural coefficient of relatedness" roughly defined as the

fraction of ideas shared in common by two people. This seems to me to be a useful extension of kin-selection theory.

D.H. Kistner has provided a fascinating discussion of social insect symbionts — the other insects and mites which are found associated in various ways with insect colonies. His discussion of the means by which these guests deceive their hosts, the extent to which some are integrated into the colony life (and others are not, but still manage to keep from being killed) and the evidence on the phylogenetic evolution of associations deserves careful study by all students of social behaviour and evolution.

M.V. Brian's chapter on "Caste Differentiation and Division of Labor" is a most imposing document. One hundred pages long with about 400 references, it documents what is known about the proximate control mechanisms for caste structure in all groups of social insects. This chapter fascinated me, both for what it left out as well as what it contained. Let me explain with reference to the concept of "colony as superorganism", a concept which appears throughout this volume. The question is: To what extent may we usefully consider a colony to be a single organism, with the queen as gonads and workers and larvae as somatic tissue geared up to aid the queen's reproduction? Hermann and Starr call it an outmoded and discarded concept. Crozier recognises that the analogy clearly breaks down at times (witness the queen-worker conflict notions of Trivers and Hare) but he is still willing to study theoretical models which explicitly assume it. He cites cogent criticism of the

concept by Mary Jane West-Eberhard; criticism directed at E.O. Wilson, whose recent book, *Caste and Ecology in the Social Insects* (Princeton University Press: Princeton and Guildford, UK) with George Oster (for review, see *Nature*, **280**, 519; 1979) certainly does treat the colony as the unit of natural selection. If these authors seem to celebrate the demise of this concept, the chapters by Wheeler and Wheeler, and Brian clearly disagree. This brings me back to Brian's chapter. An excellent discussion of the proximate control mechanisms for caste structure, the chapter never mentions the fact that the reproductive interests of workers and queen must often diverge. Where their reproductive interests do not coincide, we might expect the mechanisms controlling worker behaviour to reflect this conflict and its resolution. Brian presents several instances of caste determination which could be usefully viewed in this way — from the perspective of conflict between workers and queen.

Clearly the authors of this volume (as well as E.O. Wilson and M.J. West-Eberhard) are in conflict over the superorganism concept. If it seems unresolved at present, so be it. Contrary to much popular opinion, the selective forces involved in generation and maintenance of social behaviour of insects are not yet clear. This volume represents very well the present search for underlying principles.

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Electron image processing

Image Analysis, Enhancement and Interpretation. By D.L. Misell. Pp.305 (North-Holland: Amsterdam, New York and Oxford, 1978.) Paperback \$60; Dfl. 135. *Computer Techniques for Image Processing in Electron Microscopy.* By W.O. Saxton. Pp. 289. (Academic: New York and London, 1978.) £15.15.

THESE two books are the first single-author volumes to be devoted wholly to electron image processing, the practical importance of which has become gradually more apparent during the present decade. Despite the similarity in their titles, however, the books are very different in approach and intent. Saxton's is an advanced text, intended for readers already working in the field of digital image processing or for beginners about to immerse themselves in it. Misell's, on the other hand, is addressed to practising microscopists who wish to understand enough about image processing to know whether it could be useful to them. They may be biologists or materials scientists

and much of each chapter is therefore written as qualitatively and unmathematically as possible; furthermore, what mathematical reasoning is given is cocooned in verbal explanation and definition. Saxton's book requires the mathematical sophistication associated with a degree in physics. One might thus say that Saxton's text is written for students eager to master what he has to offer, whereas Misell's is for an audience reluctantly obliged to acquire some familiarity with this young subject.

Turning to the contents, these too are by no means the same. Misell devotes considerable space to optical methods of processing images, and the book is therefore extensively concerned with periodic specimens. His book opens with a general account of the relationship between a specimen and its electron image, followed by a chapter on optical and computer image analysis, with particular attention to helical structures. A chapter follows on image processing; superposition and filtering techniques being explained in detail. Two chapters are then devoted to contrast enhancement. The first deals with instrumental techniques: defocusing, energy-filtered images, the use of STEM, dark-field and "optical shadowing",

among others. The second describes some simple computer procedures such as subtraction of pairs of images (one highly defocused), and histogram modification. In a final chapter, the author discusses the interpretation of images of various kinds and their reliability.

Misell has gone to great lengths to present all this material simply, with the result that most of the qualitative description will not be found difficult to follow even by readers unfamiliar with the jargon of the subject. The usefulness of the more mathematical parts of the text is much more questionable, however: on p41, for example, it is assumed that the reader will need to be told that "The i which precedes η in Eq. (3.12) is used mathematically to denote a phase shift in the exponential" and that $i = \sqrt{-1}$ (though this is not the first appearance of i). A few pages later, however, he is expected to be able to cope with two-dimensional Fourier transforms in vector notation, convolutions, delta functions and the like. The section on image analysis contains much helpful guidance on indexing diffraction patterns but not really enough to learn the subject: too little for the newcomer, too elementary for the instructed. This criticism is true of much of Misell's book: as a qualitative account of electron microscopy, biased towards periodic biological specimens, it is very clear and contains an immense amount of information; but as soon as an attempt is made to become more exact, it is unsuitable for those with little mathematical background and too elementary for those who may wish to implement the various techniques. Nonetheless, it was certainly well worth making the effort to write a text at this level and much of it will be found extremely helpful.

Saxton's book covers very different ground. An opening chapter is again devoted to generalities about electron image formation, and a short chapter sets out the properties of the discrete Fourier transform. The next five chapters, the heart of the book, are concerned with the question of determining the wave function of which the recorded image indicates only the modulus, the phase being lost. This problem is still being actively explored, though less energetically than during the first few years after the publication of the Gerchberg-Saxton algorithm, the first solution proposed, in 1972. The final three chapters are concerned with the hardware and programming of image processing, and include a description of the high-level language devised by Saxton to simplify the task of image processing on a large IBM computer. However, in view of the numerous subsequent developments and the introduction of a much-improved mini-computer version of the language (SEMPER) by Horner and similar languages in other image processing centres, this material has already drifted somewhat out-of-date. It is surprising that

Misell does not deal with this topic at all, for potential users of digital techniques with no time or desire to learn much computing would surely be delighted to learn that simple languages are being devised to enable them to perform advanced processing almost effortlessly.

Saxton's book thus deals very fully with two branches of digital electron image processing: phase determination and picture handling. It is essential reading for anyone studying electron image interpretation, the phase problem in particular, and indeed, the analogous problem that arises in many other fields, optical coherence theory for example. It has the merit that not only is the theoretical support set out fully but numerical methods and computational trials are also described at length. Complicated though the subject is, Saxton has succeeded in writing a clear and highly readable account and it is unfortunate that much of the intricate work from the Groningen group led by Professor H.A. Ferwerda appeared too late to be fully integrated into the text.

Both books are well-produced, and Misell's is particularly well illustrated, many of the images having been taken specially for this volume. One minor criticism of the latter is the use of "rad" as a radiation unit as well as "radian" — as SI is adopted everywhere else, why is radiation dose not given in grays? The severest criticism that can be levelled at Misell's volume is no fault of the author, however, for the only glaring inaccuracy occurs in the preface. There, Dr A.M. Glauert, the series editor, writes "each book . . . will also be available in an *inexpensive* edition for individual use . . ." (my italics); to describe a 300 page paperback in a successful series costing \$60 as inexpensive, while a cloth-bound advanced text of much more limited appeal is priced at (only) £15.15, the series editor must somewhere have lost touch with reality.

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Quantitative study of phytoplankton

Phytoplankton Manual. Edited by A. Sournia. Pp.337. (UNESCO: Paris, 1978.)

THIS book is the latest in a series of monographs on oceanographic technology initiated by the Scientific Committee on Oceanic Research of UNESCO. Although entitled *Phytoplankton Manual* the book is almost entirely devoted to marine phytoplankton, and only four pages are specifically devoted to freshwater phytoplankton.

A group of phytoplankton workers were called together, drew up the general arrangement and content of the volume and then handed over to the editor. In his introductory chapter the editor records the problems involved in drawing together such a work and he also indicates how difficult it is to get contributors to comply with deadlines. Also in the introduction the editor expresses a hope "that the present manual will provide a description and evaluation of all current methods". This is, of course, a fine objective but an impossible one as almost every worker has some refinement or variation which is considered to be an improvement for the conditions found in a particular area. This book does, however, cover another aim of the editor in that "an attempt has been made here to cover the quantitative study of phytoplankton at all stages of research, starting even before the act of collecting a sample (i.e., planning the study) and ending somewhat after the act of

enumerating the organisms (i.e. interpreting the results)".

Consisting of a large number of short sections written by people with experience in working with phytoplankton, the methods are described in considerable detail and often compared with related methods, the book is a veritable mine of information. On the other hand because it does contain detailed descriptions of so many methods a young worker just starting in this field might be overwhelmed and find it impossible to decide which is the most suitable method to adopt. It should, however, prevent many of the initial errors which tend to detract from the value of the early results of inexperienced workers.

This, no doubt, will prove to be a valuable manual for all workers in marine phytoplankton, but I imagine a comparable one for the freshwater phytoplankton will also be required. If this book leads to a more uniform approach to the sampling, counting, statistical treatment and calculation of biomass of marine phytoplankton samples, it will have served a useful purpose.

The volume is clearly printed with few errors; it is strongly bound, although a more water-resistant cover may have been an advantage for a laboratory manual. There is an adequate list of references, a list of manufacturers of apparatus and a rather sparse index, but as the editor points out, with a chapter on "How to Use the Manual" the index does not need to be comprehensive. The price for a book of this size and containing so much practical information is extremely reasonable.

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Thermodynamics and statistical mechanics

Thermodynamics and Statistical Mechanics By P.T. Landsberg. (Oxford University Press: Oxford, 1979.) £9.75.

In this book, Professor Landsberg brings together thermodynamics and statistical mechanics to provide a coherent account of these interrelated branches of physical theory. The two subjects are treated sequentially in the text — a wise decision, as attempts to develop the two side by side usually result in the essential structures of both becoming obscured. The value of classical thermodynamics lies in what it can do without invoking microscopic models and it is best learnt by confining discussion initially to the macroscopic level. The terms of reference of Professor Landsberg's book are those of the physicist. In addition to including those topics common to most standard texts, there are chapters on fluctuations and transport properties, sections on negative temperatures and critical exponents, and introductions to irreversible thermodynamics and the theory of chemical reactions. The text also deals with subjects of current interest such as conversion and conservation of energy, black holes, and the early stages in the formation of the Universe.

Thermodynamics and statistical mechanics are both well established subjects so that any new text can only find justification if it has special merit in excellence of exposition or in its choice of emphasis. This book has some attractive features: sections containing core material are distinguished so that the reader may restrict his study to the central development if he wishes; worked solutions are given to the problems; and there are references to papers and comments on a selection of other books so that topics of interest may easily be pursued. The text is at its best (and it is then very good) in some of the sections dealing with particular applications; examples are the expanding universe, negative temperatures, and energy conversion. The general exposition, however, is unsatisfactory. Professor Landsberg states that he is fascinated by the relationship between physics and mathematics; this fascination, however, has unfortunate consequences in the text. The language used in the basic development of the subjects is highly formal, and mathematical structure frequently precedes physical explanation of what the structure is being erected for. As a result, one sometimes has the impression that physical results are eventually produced rather like rabbits from a conjurer's hat. It was diverting to discover that the phase rule is the same as the Euler formula relating the number of vertices, edges and faces of a

convex polyhedron; but knowledge of the fact gave me no new insight into the physical world. Is it necessary or even helpful to the typical student to be faced with density matrices, Hessian determinants, Pfaffians and the signs of symbolic logic? It is, in fact, difficult to imagine the kind of student who, coming with little previous knowledge of thermodynamics and statistical mechanics, could find this a good book through which to learn them.

There are other grounds for criticism. It is surprising to find no mention of thermodynamic temperature, no formal definition of the kelvin, no mention by name of the principle of equal *a priori* probability. It is confusing to find both chemists' wavenumber ($1/\lambda$) and physicists' wavenumber ($2\pi/\lambda$) used in different parts of the text. It is disconcerting to find important results such as the Maxwell Relations or general equilibrium conditions derived in problems at the ends of chapters. It is irritating to find the Carnot cycle being used in the text

before it is defined, again in a problem.

The book is also disappointing as regards its production. Oxford University Press has chosen, presumably in order to keep down costs, to use photographic reproduction of typescript. This can give a text of entirely acceptable appearance providing that the typescript is meticulously prepared. Here, however, alterations are frequently all too visible; in some cases corrections have been made and symbols inserted in ink by hand; and, in at least one place, there is a gap in the text where words have been deleted.

There is some very good material in Professor Landsberg's *Thermodynamics and Statistical Physics*; but the style of his exposition will make it inaccessible to most students. It is a book to be dipped into by those who already know the subject, not a book to learn it from.

C.J. Adkins

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Obesity models

Animal Models of Obesity. Edited by M.F.W. Festing. Pp. 258. (Macmillan: London, 1979.) £17.50.

SYMPOSIA proceedings are all too often fabulously expensive and hopelessly delayed in publication, so that their only logical home is the library of a well heeled institute of scientific history. This volume — subtitled *MRC Laboratory Animals Centre Symposium No 1* — is the exception to that rule. Though not cheap, it is, by today's standards reasonable in price, and, to judge from its plastic dustcover, the publishers see its home as much in the laboratory as the library. And there it will be found, for it is an authoritative text which covers a major experimental field in nutrition and is likely to be the only repository for a great deal of useful technical information, like Derek Miller's recipes for energy-dense diets, or David Lovell's outstandingly lucid guide on husbandary techniques for colonies of genetically obese rodents. But it is more than a technical guide, it is a unique review of a fast growing field, and one in which biochemists, geneticists, immunologists and nutritionists all meet, to exchange information and to generate ideas.

The book succeeds in giving the impression of interaction, both because of Dr Festing's careful editorial guidance, reflected in, for example, the frequent cross references between chapters, and because the symposium on which it was based was a fairly parochial, virtually all-British affair, in which all the contributors have closely followed each other's work.

This parochiality is a trait which I find strengthens, rather than weakens, the feeling of a debate, and it is made possible by the fact that Britain is a leader in obesity research.

The symposium was originally entitled *Genetic Models of Obesity in Laboratory Animals* but the change in title here is more than justified by the extent to which a number of authors, particularly Miller, Stock and Rothwell, James *et al.* and Garrow, have turned away from the simple genetic models such as the *ob/ob* or *db/db* mouse and the Zucker rat, and studied 'dietary-induced obesity': that in which no simple genetic component can be discerned. It may be, as many authors claim, that these are more appropriate models for the study of obesity in man, which is, after all, the justification for all the work.

At the same time, however, the simple genetic models have a scientific attraction which increases with every new defect they are shown to possess. This volume brings out the multitude of differences that the single Mendelian recessive *ob/ob* gene gives rise to, and in reading it, it is difficult to resist playing the game that so obviously fascinates many of the authors: to fit this metabolic mélange into the single enzyme defect that genetical orthodoxy requires. The man who solves that puzzle will perform a scientific feat that is in no way diminished by the fact that it will not provide a cure for obesity. It is a prize which is there for the grabbing, and anyone who wants to pursue it could do no better than to start with this eminently readable text.

John Rivers

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Chemistry of metal complexes

Metal Complexes in Organic Chemistry. By R.P. Houghton. Pp.308. (Cambridge University Press: Cambridge, 1979.) Hardback £21; paperback £7.50.

I ENJOYED reading this book; it is written in a concise manner and is a mine of useful information. Its complex chemistry is a little thin in places, but perhaps the dose is correctly adjusted to the organic chemists whom the book addresses. The author has done a remarkably fine job in bringing together and systematising the vast variety of interesting organic reactions made possible or more specific, catalysed, inhibited or altered in direction by the action of metal containing compounds.

The book is aimed at "advanced undergraduates, graduates at the start of a research career in organic chemistry and practising organic chemists who wish to learn more about the chemistry of metal complexes". It is an excellent book for that purpose, and must represent an enormous amount of work on the part of its author. Its main deficiency is a lack of reference to recent primary literature, obviously a matter of policy, but it does give reference to some 125 well chosen reviews.

In 308 pages it is quite impossible to catalogue all of the organic reactions mediated by metal complexes. Nevertheless the author has made a very comprehensive survey of reaction types -except olefin polymerisation, but Ziegler polymerisation of olefins does receive brief mention (page 244). Each type of organic reaction is profusely illustrated by examples, well set out in display formulae. This means that a mere inorganic chemist such as your reviewer was never at a loss to understand the nature of the organic materials mentioned in the text, even in the very few cases where the name did not convey to him the essential chemical structure intended - for example, "methylmagnesium carbonate" on page 104 is not the probably hypothetical MeMgO.CO.OMgMe , but methyl methoxymagnesium carbonate, MeOMgO.CO.OMe .

The first and second chapters are concerned with general principles of complex chemistry in their application to the explanation of organic reactions mediated by metal complexes. They are very useful chapters with emphasis on electronic effects relating mainly to σ - and π -donation by ligands, π -donation by the metal atom, relative metal-ligand affinities, steric effects, and coordination saturation. They indicate how these affect the egress of organic reagents to the metal, whether as strongly or weakly bound ligands, and how the reagents are polarised or otherwise activated to chemical attack. The rationalisation of the complex

chemistry which is desired for the purpose of this book is good. It is only deficient in taking no account of ligand field theory, especially to explain the stabilisation of the higher valent states of such metals as iron, cobalt and nickel, for example, by thio and non- π -donor ligands (page 47) and in the explanation of differences between the light and the heavier transition metals. Some short outline of ligand field effects, with a reference to appropriate reviews by the late Sir Ronald Nyholm or L.E. Orgel would have been appropriate here.

The types of reactions covered in the text are much too numerous to mention in this review. They range from the venerable Fredel-Crafts alkylations catalysed by aluminium chloride to the recently developed homogeneous hydrogenation, carbonylation and decarbonylation catalyses based on the heavier Group VIII metal complexes with tertiary phosphines, carbon monoxide (and so on) as ligands. The use of metal complexes in organic chemical synthesis ranges from the simple separation and purification of organic products as ligands, through mediation in organic reactions by shifting equilibria in favour of the desired product, through ester hydrolyses, bond cleavages especially of the CH bond, substitutions, isomerisations especially of alkenes,

hydrogenations, additions and eliminations of various sorts. They are all there, with a strong emphasis on explanations in terms of established physical-organic chemical mechanisms. These are clearly set out and copiously illustrated.

Although I sense that the author lacks some feel for inorganic chemistry, for example, as in his assertion that aluminium chloride exists as Al_2Cl_6 molecules in the solid state (page 127) and his formulation of $[\text{ReCl}_3(\text{NMe})(\text{PEt}_2\text{Ph})_2]$ with a non-bonding electron pair on the nitrogen atom (page 96), the book is a text which I strongly recommend. It is well researched, organised and printed. It summarises beautifully the types of interesting organic reactions which occur on metal complexes and has a very useful 10-page index. At present-day prices it is good value at £21. I congratulate the author and his publisher on a unique and well produced book in an area of chemistry which has been surprisingly slow in catching the imagination of organic chemists.

Joseph Chatt

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Neurophysiological offerings

Studies in Neurophysiology. Presented to A.K. McIntyre. Edited by R. Porter. Pp.440. (Cambridge University Press: Cambridge and New York, 1979.) £32.50.

THIS book, as it proclaims on its cover, is a collection of offerings from his former collaborators and students to a much-loved and respected Australian neurophysiologist, Professor A.K. McIntyre. Professor McIntyre was successively head of the Department of Neurophysiology at Otago and the Department of Physiology at Monash, which quickly grew into a hugely successful venture. The success of these two departments can quickly be appreciated by a glance down the star-spangled list of contributors to this book, many of whose names would be recognised (with enthusiasm, one hopes) by any undergraduate student of neurophysiology.

As an offering to Professor McIntyre, it must have given great pleasure; but how does it rate as a book about neurophysiology? The main difficulty in compiling such a book for publication as opposed to presentation is obviously to keep a coherent thread running through it, and to achieve consistency of style among the many contributions. Many of the

individual contributions are, as one would expect, excellent. There are, for example, clear and elegant reviews of such topics as the vesicle hypothesis (Katz), the mechanism of excitation of carotid body chemoreceptors (Eyzaguirre & Fidone) and the functional significance of changes in extracellular potassium concentration in the central nervous system (Somjen). Other authors have chosen to give much more specialised and technical accounts of particular research projects (for example, Laporte on muscle spindle innervation, and Mubbard on the effects of angiotensin on brain neurones).

Towards the end another abrupt gear change takes place with a long and heady philosophical article by Trevarthen called 'Self-organising systems in psychobiology' (Typical headings: Is a mind biology possible?; Anatomies of imagery and intention; Normal duplicity of brains) which is followed by an essay on Claude Bernard and the *milieu intérieur*.

Though good in places, without a doubt, this collection amounts overall to an indigestible motley which might have been better kept as a private retirement present than expensively dressed up for publication. Unless you are in a sentimental mood, the outlay would be hard to justify.

H.P. Rang

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obituary

Samuel A. Goudsmit, 1902-1978

On 4 December 1978 the world of physics lost one of its most unusual and interesting members when Professor Samuel A. Goudsmit died of a heart attack on the campus of the University of Reno, Nevada. His career was as varied as the times in which he lived; and the changes in science and scientists are accurately mirrored in his actions and his life.

Samuel A. Goudsmit was born in The Hague, The Netherlands, on 11 July 1902; he lived and grew up in a middle class family of merchants. At an early age he showed extraordinary mental agility. He was exceptional in solving puzzles of all kinds. When a young man in high school Goudsmit happened to get a hold of a cheap spectroscope (it presumably belonged to his older sister) and after examining the brilliant spectral colours, Goudsmit was hooked, and spectroscopy and the physics going with it became not only his interest, his hobby and his profession, but a lifelong passion.

Because of his interest first in spectroscopy and later in physics as a whole he started his studies at the University of Amsterdam. After receiving an intermediate degree he went on to obtain his PhD at the University of Leiden. Although Goudsmit's performance was brilliant (with some notable exceptions when he had no interest in the subject) he was considered somewhat of a failure by his family. Physics at that time was a calling, not a profession; with the certainty that there would be no jobs after completing the degree, it was reasonable that Goudsmit's family had a difficult time understanding his devotion to physics. In spite of his unquestioned early successes he was considered as a bit of a misfit.

At the university Goudsmit did well — in physics he did exceptionally well. He was only 19 years old when he pointed out in a paper, (*Naturwiss* 9, 995, 1921), that the Sommerfeld formula for x-ray doublets was equally valid for alkali doublets. The knowledge, but perhaps even more, the scientific maturity necessary for that observation is highly unusual for a beginning student.

In Leiden, Goudsmit came under the spellbinding influence of Ehrenfest. Ehrenfest was impressed by Goudsmit's talent and interest. The combination of Ehrenfest's insistence on precision and clarity and Goudsmit's natural flair for physics led to Goudsmit's surprisingly rapid development as a scientist. It is therefore understandable that when



another one of Ehrenfest's students, George Uhlenbeck, returned to Holland (from a sojourn in Italy) Goudsmit was given the task of bringing Uhlenbeck's knowledge of contemporary physics up to date.

It was in discussions between these two young men, still students, in 1925 that they introduced the notion of "spin". It may well be that by now the familiarity with this concept has blurred the unusual character of their proposal. It took a great deal of knowledge and even more courage to systematically explore and analyze the consequences of this idea. Although Ehrenfest continually encouraged them; the novelty and boldness of this step should certainly be kept in mind.

In 1926 both Uhlenbeck and Goudsmit were offered instructorships at the University of Michigan. There Goudsmit continued his work on the analysis of interpretation on atomic spectra. The field was summarized in an important text on atomic physics written together with Linus Pauling, *The Structure of Line Spectra* (McGraw-Hill, 1930).

Although Goudsmit had been extremely successful in the early phase of his scientific life it would be wrong to say that he was deliriously happy. His unhappiness and moodiness became more pronounced as time went on. There were many reasons for that: one was that (in part, due to his own researches) the field of physics in which he was most expert had reached a certain degree of completion; as such it was no

longer in the center of interest. Although Goudsmit later worked in a number of different areas in physics, he never again felt as attuned and in control of a field, as he did in atomic spectroscopy. Although enormously talented Goudsmit often had the feeling that he did not possess enough intellectual technology to function effectively in physics. He had never been terribly interested in mathematics. (He used to say very frequently that he could only understand 2 by 2 matrices and nothing more. This is probably not true, but if it were he certainly made the absolute maximal use of the mathematics he controlled.) Goudsmit often expressed concern about his lack of mathematical ability. He felt that in order to advance in experimental physics a rather detailed understanding of accelerating machinery was essential — that knowledge he did not have either. This made it difficult to choose an effective new research area. There can be little doubt that the dramatic ending of Ehrenfest's life and the calamitous European political situation of the 1930's had a profound effect on Goudsmit. This affected everybody and in particular a sensitive person like Goudsmit. His scientific productivity obviously suffered and this in turn added to his general dissatisfaction with himself and with the world.

It cannot come as a surprise that when the Second World War broke out Goudsmit felt honour bound to take as active a part as possible. He first joined the MIT radiation laboratory and did some work on radar. Later he became the Chief Scientific Officer of the Alsos Project. This was a remarkable scientific intelligence mission; its purpose was to find the extent and status of German atomic weapons research. The results of his mission are written up in a book entitled *Alsos* (Sigma Books, London 1947) which unfortunately is out of print. It describes the mood and the atmosphere of wartime research in Germany. It is also intensely personal and written with great sensitivity — even pathos as when Goudsmit learned that his parents had been deported to an extermination camp. Only those who have suffered the trauma of tragedy and guilt which accompanies the total destruction of a family will fully appreciate the depression that is produced and only they will understand the extraordinarily painful struggle needed to keep going and in fact start anew.

After a short sojourn at Northwestern

University, 1946-1948, Goudsmit joined the Brookhaven National Laboratory in 1948. His interest and devotion to physics was as intense as ever. From 1952 to 1960 he was Chairman of the Brookhaven Physics Department; but equally important was his influence as Managing Editor of the *Physical Review*. He initiated the first letters journal, *Physical Review Letters*, still the most prestigious letter journal in physics.

Goudsmit received many honours, most of which were in connection with the discovery of spin. As expected he shared many of the honours with the codiscoverer of spin, George Uhlenbeck. They received the Research Corporation Science Award (1954), the Max Planck Medal of the German Physical Society (1965), the Karl T. Compton Award for Distinguished Statesmanship in Science, American Institute of Physics (1974) and the National Medal of Science (1977). There was also a commemorative meeting about spin and its discovery at the American Physical Society Annual Meeting in January 1976. Goudsmit and Uhlenbeck got their PhD degrees on the same day, 6 July, 1927. To express the appreciation and admiration of the Dutch scientists a special symposium was held to honour the 50th anniversary of their PhD degrees on 6 July 1977. It was a two day symposium with two highlights: the public highlight occurred when Goudsmit and Uhlenbeck received from the Dutch Government a very high decoration in recognition of their great accomplishments. This was not only a signal honour but a diplomatic feat as well, for such decorations can only be granted to Dutch citizens and Goudsmit and Uhlenbeck although born in Holland, were both naturalized American citizens.

But probably the more impressive and certainly the more touching celebration came when I walked with Goudsmit to the room where 50 years before he had taken his PhD examination. In that old room at the University of Leiden it is a tradition that after passing an examination the students write their names on a wall giving the date and type of examination. Goudsmit had bought a "magic marker" for the occasion; together we walked into the room where he had taken the examination (over the mild objection of the doorman). On the wall were hundreds of signatures. After some searching we found Goudsmit's signature for the right exam at the right time. Right above it Goudsmit wrote his signature with the black magic marker and added "Golden Jubilee of the PhD, July 6, 1977". After that we very quietly walked back. It was very clear to Goudsmit this was a significant and important event.

With the passing of Samuel Goudsmit the world of physics, indeed the whole world has lost a person of great sensitivity, understanding and talent. He was a man of fanatical honesty who detested circumlocution and vagueness. He was the exact

opposite of pompous. His standards in physics and in life were extraordinarily high. He made corresponding demands on those around him — on his colleagues, on physics, and most certainly upon himself. That does not make for a terribly easy life, it does not make for a uniformly happy life, but it does mean that one can say with honesty and conviction — it mattered to the world that Samuel Goudsmit lived.

Max Dresden

R.N. Pryor

ROBERT NELSON PRYOR died of a heart attack in London on 14 July 1979, a few days short of his 58th birthday. He was at the peak of his powers and authority as a leading figure in the mining world.

Born in India while his father, a prominent mining engineer, was working in the Kolar Gold Fields, he was educated at Oundle and entered the Royal School of Mines, Imperial College, in 1939. After a year in which he played rugby for the RSM and rowed in the college first VIII, he joined the Royal Engineers and served in India and then with the Chindits in Burma. He finished the war with the rank of Captain, married the daughter of a New Zealand mining engineer, and returned to the College to obtain a first class honours degree in mining engineering in 1948.

His early career had a curious six-yearly cycle. Six years in the army and, when on graduation he went to Rio Tinto Mines in Spain, he spent six years in junior management before being made Chief Mining Engineer — a position which he occupied for six years. He then returned to the London office of Rio Tinto Zinc from where, for six years, he examined new mining projects including potash, lead-zinc, copper, nickel and pyrite in various parts of the world. In 1966 he was invited by the Spanish directors to become General Projects Manager of Rio Tinto Patiño, and in this post he supervised the launching of Cerro Colorado, the largest gold and copper mine in West Europe, and started construction of a new copper smelter and refinery at Huelva. His long and important contributions to Spanish mining did not go unrecognised, for in 1977 he became the first foreigner to receive honorary membership of the Asociación de Ingenieros de Minas de España.

In 1968 he was appointed Professor of Mining at Imperial College and brought back to his old school a wealth of experience in modern mining practice and management. As a teacher, his particular interests were in mineral production management, the design of large open-cast operations and in countering the environmental effects of mining past and future. He was much in demand as a consultant and, as his reputation grew, he became increasingly involved in recalcitrant problems, world-wide, as both technical adviser and arbiter. This

heightened authority and experience was used to the benefit of his students — both undergraduate and postgraduate — and of his staff, many of whom were involved with him in the important, topical problems on which he was engaged.

In 1974 he became Head of the Department of Mineral Resources Engineering and, about the same time, began to assume the influence in professional engineering circles that culminated in his presidency of the Institution of Mining and Metallurgy and membership of the Executive Committee of the Council of Engineering Institutions. He did not spare himself, contributing in full to both organizations and serving as chairman and member of a large number of committees. Because he firmly believed that the engineering professions must come closer together, he was also an active Fellow of the Institution of Civil Engineers. He exemplified his conviction by organizing the Tunnelling '76 and Tunnelling '79 International Symposia — highly successful meetings which involved all three Institutions.

With his Presidential Address to the IMM, "Towards a Minerals Policy", delivered in June 1978, Bob Pryor embarked on what was to be his final project — to persuade the professions, industry and government of the need to take active, positive steps to ensure our vital mineral supplies. He followed this up with an informal meeting between government officials and representative mining and metallurgical experts in March 1979, and was one of the prime movers in arranging the National Symposium on the Availability of Strategic Minerals to be held in November of this year. His final contribution was an address entitled "Ensuring Access to Vital Supplies for Britain", which I read to the Parliamentary and Scientific Committee a few days after his death.

There was no badinage, no easy small-talk and certainly no flattery; but to his friends and those who enjoyed his confidence, he was the best of companions — warm and frank, interested and sympathetic, forthcoming and always sincere. Although his own standards were so high, he was in no way smug. He neither expected nor wanted others to share his views except by conviction. He was stubborn but open to reason. He was firm but generous. He had pride, but no conceit and, indeed, he was unduly diffident in some respects. He was humane and tolerant — up to a point — but he could not tolerate dishonesty. If, to these characteristics, you add intelligence, industry, an acute critical faculty, determination and a deep sense of responsibility, you have leadership. If you add courage and absolute integrity, you have a model for a complete professional engineer.

He is survived by his wife Patricia, three sons and a daughter. M.G. Fleming